THE JOURNAL OF EXPERIMENTAL MEDICINE

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME TWENTIETH

WITH FORTY PLATES AND FIFTY-THREE FIGURES IN THE TEXT

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1914

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THE NEW ERA PRINTING COMPANY
41 NORTH QUEEN STREET
LANCASTER, PA.

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PRESENT CONDITION OF A STRAIN OF CONNECTIVE TISSUE TWENTY-EIGHT MONTHS OLD.*

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 1 AND 2.

In previous articles[1] it was shown that connective tissue could be kept outside of the organism in a condition of permanent life. The purpose of the following experiments was to determine the present condition of a strain of connective tissue which, after having undergone 358 passages, has now reached the twenty-ninth month of its life *in vitro*. The strain of connective tissue was derived from a piece of heart extirpated on January 17, 1912, from a chick embryo seven days old. The fragment of heart pulsated for 104 days and gave rise to a very large number of connective tissue cells. These cells multiplied actively during the last two years, and produced a large amount of connective tissue. At present, a great many cultures are obtained from the strain every week.

The dynamic condition of a tissue is manifested by the rate of its growth. The increase in the volume of a fragment of connective tissue can be measured with comparative accuracy. For this the following technique is used. A fragment of tissue is removed from a culture, washed in Ringer solution, and placed in a new medium. It soon becomes surrounded with a ring of new tissue. After forty-eight hours the width of this ring is measured with a micrometer. Under the ordinary conditions of the experiment the thickness of the new tissue is more or less uniform, and its total volume can therefore be estimated fairly accurately by its superficial size. The fragments of tissue usually double in forty-eight hours. But their rapidity of growth is subject to fluctuations dependent upon the character of the medium and upon the condition of the tissue at the time that it is placed in the medium. When these conditions were favorable the ring of new tissue attained, during

* Received for publication, May 10, 1914.

[1] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516. Ebeling, A., *idem*, 1913, xvii, 273. Carrel, A., *idem*, 1913, xviii, 287.

the last few months, a width of 2 to 2.8 millimeters (figures 1 and 2). A comparison of the amount of tissue produced by a given culture in forty-eight hours this year with that produced in the same time by the same strain of cells a year ago shows that the activity of the strain has increased. Last year the width of the ring of tissue produced in forty-eight hours around the fragments of connective tissue was only 1.5 or 1.8 millimeters.

This increase in the rate of growth is made more apparent by the following experiment. A piece of heart extirpated from a chick embryo eight days old, and a fragment of connective tissue at the beginning of the third year of its life *in vitro* were placed in the same culture medium. After forty-eight hours it was seen that the tissue which had become adapted to the life *in vitro* had increased much more rapidly than the fresh tissue (figure 3). Nevertheless, the tissue adapted to the life *in vitro* was derived indirectly from a fragment of heart extirpated more than two years ago from an embryo seven days old. Thus it is conclusively shown that the proliferating power of the strain has in no wise diminished. Nevertheless, it would be imprudent to conclude from this fact that it has augmented, as the greater rate of increase of the tissues may be due, not to an augmentation of the proliferating power of the cells, but to an improvement in the details of the technique.

Moreover, the fact remains that during the third year of independent life the connective tissue shows greater activity than at the beginning of that period, and is no longer subject to the influence of time. If we exclude accidents, connective tissue cells, like colonies of infusoria, may proliferate indefinitely.

EXPLANATION OF PLATES.

Plate 1.

Fig. 1. A fragment of connective tissue extirpated from a culture of the twenty-eight months old strain, one hour after the passage.

Fig. 2. The same tissue, forty-eight hours after the passage.

Plate 2.

Fig. 3. In the same medium were placed a piece of heart from a chick embryo eight days old (A), and a fragment of connective tissue, No. 8860, which had lived for more than two years outside of the organism (B). The photograph shows the amount of tissue produced in forty-eight hours by both fragments.

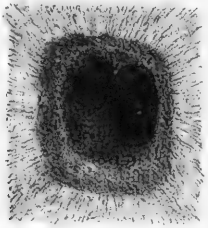

FIG. 1.

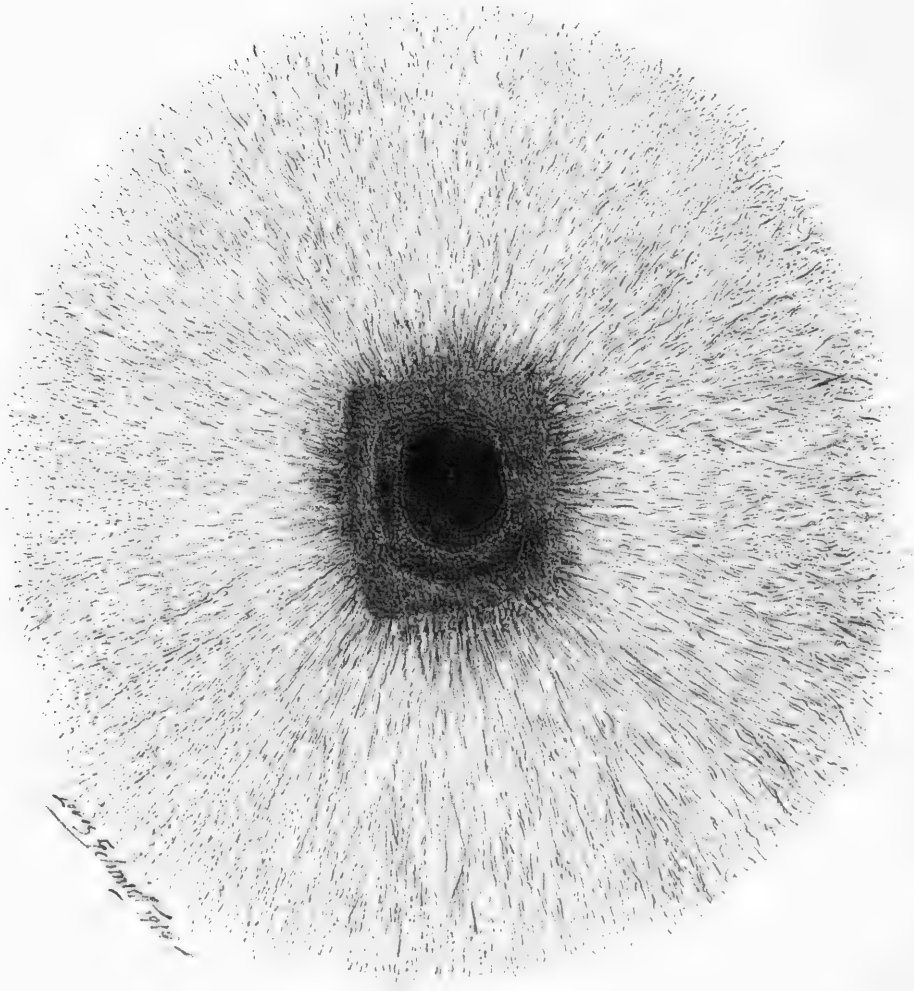

FIG. 2.

(Carrel: Condition of Strain of Connective Tissue.)

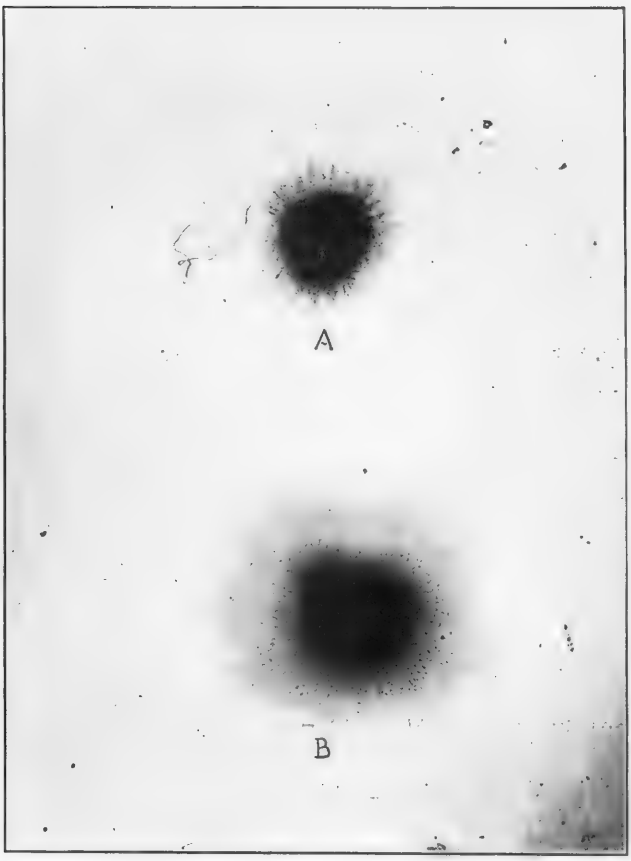

FIG. 3.

(Carrel: Condition of Strain of Connective Tissue.)

PATCHING AND SECTION OF THE PULMONARY ORIFICE OF THE HEART.*

By THEODORE TUFFIER, M.D., and ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The purpose of these experiments was to develop a technique by means of which the pulmonary orifice could be enlarged. The operation consisted in suturing to the anterior side of the orifice a venous patch which permitted an increase in the circumference of the orifice after the arterial wall had been incised.

TECHNIQUE.

The animals were etherized according to the Meltzer-Auer method. The thoracic wall was shaved with sodium sulphide and sterilized with iodin, and the animal was put on an electric warming pad. The thoracic cavity was then opened on the left side by a large transverse incision which was kept widely open by means of a Gosset retractor. The operating field was walled off by means of a technique precluding the occurrence of pleurisy, which has already been described.[1] The pericardium was opened through a long incision made on the right side of the phrenic nerve at about two centimeters' distance from the nerve, and the anterior part of the heart and of the pulmonary artery was exposed.

The position of the valves was easily located. The wall of the pulmonary artery is thinner at the level of the sigmoid sinuses and its color is slightly darker. This point was selected for the lower end of an incision of about 1.5 centimeters made through the arterial wall above the anterior sigmoid valve. It was found afterwards that this incision was too short, and that when it was longer it produced a very marked pulmonary insufficiency. In order to be more efficient and less dangerous, the section of the orifice should be

* Received for publication, May 10, 1914.

[1] Carrel, A., *Surg., Gynec. and Obstet.*, 1914, xix, (in press).

made at the point of insertion of the anterior and left sigmoid valves on the arterial wall. After the location of the incision had been selected the patch was applied on the anterior wall of the pulmonary artery and of the heart. This patch was usually a piece of vena cava which had been preserved in cold storage for a few days. Pieces of artery could also be used, but as the arterial wall is thicker and harder than the venous wall it was more difficult to fix the patch to the pulmonary wall without danger of leakage. The shape of the patches was generally rectangular, about 2.5 by 2.5 centimeters. The patch was fixed to the pulmonary artery and to the heart by four stitches. The stitches of the lower and upper sides of the rectangle were about 2.5 centimeters distant from each other. The right and left sides of the patch were placed in such a way that the distance between the right and left lines of suture was only about 1.5 centimeters. As the width of the flap was 2.5 centimeters this allowed of one centimeter's increase in the circumference of the pulmonary orifice after it had been cut. The right upper and left sides of the flap were fixed to the anterior wall of the pulmonary artery by a continuous suture. Between the lower end of the flap and the wall of the heart there was a free space through which the incision of the orifice could be made. For this purpose special scissors were constructed, composed of one long sharp blade and one short blunt blade. The arterial wall was perforated with the long blade of the scissors just at the level of the pulmonary orifice. The long blade was pushed into the lumen of the pulmonary artery, and the wall was cut by introducing the short blade deep under the flap. There was an immediate escape of dark blood, the scissors were quickly removed and at the same time the index finger of the assistant compressed the flap against the opening made with the scissors, thus arresting the hemorrhage. Then the lower side of the flap was rapidly united to the cardiac wall by means of a continuous suture. This stage of the operation was generally made without interrupting the circulation through the heart. The operation was facilitated by interrupting it for one or two minutes; but as the clamping of the pedicle of the heart is always somewhat dangerous, this procedure was not often used. After the suture had been completed slight compression with a

sponge was applied on the flap and on the lines of suture. If there was still some leakage after a few minutes, complementary stitches were added. The pericardium was never closed before the line of suture was absolutely secure against the escape of blood. After suture of the pericardium, the thoracic cavity was closed by the ligation of two ribs and by three or four planes of suture. Afterwards the dog was dressed and cared for by the customary methods.

EXPERIMENTAL.

PATCHING OF THE PULMONARY ARTERY.

The experiments were performed on eight medium sized dogs. The technique for the first four operations was in its developmental stage and differed slightly from that used for the last four operations.

Experiment 1.—Black female dog, medium size. Oct. 23, 1913. Etherization by the Meltzer-Auer method. Transverse thoracotomy. Ligature of both mammary arteries and section of the sternum. Opening of both pleural cavities. Incision of the pericardium. On the anterior part of the pulmonary artery and of the cardiac wall was placed a fragment of aorta taken twenty-four hours previously from a fresh cadaver of a human fetus and preserved in cold storage. The upper and the lateral sides of the patch were fixed to the pulmonary wall by a continuous suture. Then the pedicle of the heart was clamped. A small knife was introduced under the patch and the wall of the pulmonary artery was cut. The incision was too short and too high. Next, the lower side of the patch was sutured to the cardiac wall, and the clamp was removed after the circulation had been interrupted for two minutes. Massage of the heart. After a few minutes the contractions were normal. Closure of the pericardium and of the thoracic cavity. No shock. After the operation the animal remained in normal condition. May 5, 1914. Animal in excellent health.

Experiment 2.—White dog, medium size. Oct. 26, 1913. Etherization by the Meltzer-Auer method. Opening of the thoracic cavity by the same method as that used in experiment 1. The patch was made of a fragment of dog's aorta preserved for twenty-four hours in cold storage. The clamping of the pedicle of the heart and the suture of the flap were performed as in the previous operation. The section of the wall of the pulmonary artery was made with fine scissors at the level of the insertion of the sigmoid valves. After the clamp was removed fibrillary contractions appeared and the animal died. An examination of the specimen showed that the wall of the pulmonary artery was cut as far as the insertion of the anterior valve. The opening was completely protected by the patch, but a small branch of the coronary artery had been taken up by a stitch. This was possibly the cause of the fibrillary contractions. The incision should have been made further to the left side of the artery, in order to cut the orifice at the point of insertion of the left and anterior valves.

Experiment 3.—Black and white bulldog, medium size. Oct. 31, 1913. Etherization by the Meltzer-Auer method. Transverse thoracotomy on the left side without section of the sternum and without ligation of the mammary arteries. A piece of dog's jugular vein preserved for twenty-four hours in cold storage was placed on the pulmonary artery. The lateral and the lower sides were fixed to the arterial and cardiac wall by a continuous suture. Then the wall was cut with scissors as far as the insertion of the sigmoid valves. Hemorrhage was prevented by compressing the flap to the opening with the index finger. Next, the upper side of the flap was sutured to the arterial wall. No massage of the heart. Normal pulsations. Closing of the pericardium and of the thoracic cavity. Two hours afterwards the animal was in excellent condition. May 5, 1914. Dog normal.

Experiment 4.—Female bulldog, medium size. Nov. 3, 1913. The same technique was used as in experiment 3. The patch was taken from a piece of vena cava preserved in cold storage since Oct. 27. It was sutured without the heart being clamped. No shock. The animal remained in good condition after the operation. Nov. 6. Animal ill. Nov. 8. Animal died.

Autopsy.—Purulent pericarditis. The space between the flap and the arterial wall was filled by a clot. The incision had healed. No fibrin or thrombus in the lumen of the vessel. As the incision was too short there was no increase in the circumference of the orifice.

Experiment 5.—Black and white male fox-terrier. Nov. 4, 1913. Etherization by the Meltzer-Auer method. Opening of the thoracic cavity and the pericardium with the same technique as that used in experiment 4. The patch was made from a piece of vena cava preserved in cold storage since Oct. 27. It was fixed on three sides and the anterior wall was opened with a cataract knife without any interruption of the circulation of the heart. Then the fourth side was sutured and the operation completed by the ordinary method. After the operation the dog remained in excellent condition. On Nov. 19 the animal had completely recovered. Strong diastolic murmur could be heard. May 1, 1914. Animal in excellent condition. The murmur had almost completely disappeared.

Experiment 6.—White female bulldog. Nov. 11, 1913. The technique used was identical with that of experiment 5. On Nov. 19 no murmur was audible. The animal remained in excellent condition, and is completely normal on May 5, 1914.

Experiment 7.—White male bulldog. Nov. 26, 1913. Etherization by the Meltzer-Auer method. The technique was identical with that used in the last two experiments. The section of the wall was made with special scissors composed of a long sharp blade and a short blunt one. The animal remained in excellent condition after the operation and is still normal on May 5, 1914.

Experiment 8.—White female bulldog. Dec. 2, 1913. Etherization by the Meltzer-Auer method. Technique identical with that used in the last three operations. The patch was made from a piece of jugular vein preserved in cold storage since Nov. 28. The animal remained in excellent condition and is still normal on May 5, 1914.

RESULTS.

The results of these operations must be considered from two standpoints: the danger to the life of the animal, and the modifications of the pulmonary orifice.

When the technique previously described was carefully followed, the operation was of little danger to the life of the animal. Although these operations were in the developmental stage, nevertheless only two animals died of the eight that were experimented upon. One of these died on the operating table from fibrillary contractions, probably due to the fact that a branch of the coronary artery had been taken up by one of the stitches. A second dog died of pericarditis a few days after the operation. It is probable that neither of these complications will occur again, since a better way of handling the heart has been found. In the six other instances the animals sustained no shock after the operation, remained in good health, and were still normal more than six months after the operation. The operation will be much less dangerous in the future, because the details of the technique have now been completely established.

The changes produced in the anatomy of the pulmonary orifice were examined on two specimens. In experiment 2 the dog died of fibrillary contractions on the operating table. The specimen showed that the incision had been too short and was located on the right side of the artery. The operation as it was performed was insufficient as well as dangerous. It was insufficient because the circumference of the orifice was not increased; and it was dangerous because the flap was fixed to the cardiac wall at a site where there were many branches of the coronary arteries. In experiment 3 the animal died of pericarditis. The flap had taken, but the incision was too short. The examination of the flap and of the incision demonstrated that no thrombosis had taken place, and that the patch of vein in contact on one side with dark blood and on the other with the pericardium, although in an unfavorable condition of nutrition, did not undergo necrosis. In the clinical examination of the six other animals it was found that only one presented a diastolic murmur a few weeks after the operation; that is, in one instance only was pulmonary insufficiency obtained. The lack of insufficiency in the other cases was due to the fact either that the incision of the pul-

monary artery was located too high and was too short, or that the flap was fixed too tightly to the arterial wall and did not allow of the dilatation of the orifice after it was incised. These details had to be learned in the course of the experiments, and it will be an easy matter to make an incision and patching which will permit a real dilatation of the orifice to take place. The incision must be about two centimeters in length, half being on the pulmonary artery and half on the ventricle, and it must cross the orifice at the point of junction of the left and the anterior sigmoid valves. The incision of the orifice and the suture of the fourth side of the flap will be made after the pedicle of the heart is clamped.

The six surviving animals are still in good health more than six months after the operation. In experiment 5 the diastolic murmur almost completely disappeared.

CONCLUSION.

These experiments show that it is possible to perform an operation, the object of which is to increase the circumference of the pulmonary orifice without involving much danger to the life of the animal. It is probable that operations of this type may come to be employed in the treatment of stenosis of the pulmonary artery in man.

EXPERIMENTAL OPERATIONS ON THE SIGMOID VALVES OF THE PULMONARY ARTERY.*

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 3 TO 5.

The purpose of the following study was to ascertain whether and to what extent intracardiac operations could be performed with safety. The technique that has been described and employed by various experimenters and surgeons is unsatisfactory, because it involves the introduction of sharp instruments into the cavities of the heart, without the control of the eye or the finger. Thus far no operations have been devised which permit, for example, the cauterization of infected valves, the suture of the foramen ovale, or of two valves in a case of insufficiency, and still other plastic operations. The operations mentioned form a different class from those which have so far been performed, because they involve the stoppage of the circulation through the cavities of the heart and the passage of air into the cardiac cavities, and require great speed of execution. Hence it is hardly to be expected that a technique making possible and safe such operations as these will be perfected soon. It may even be regarded as extremely doubtful whether this class of operations may ever be applicable to human surgery. And yet their future cannot be predicted, and I have, therefore, attempted to develop methods for the execution of these operations. For the preliminary studies the sigmoid valves of the pulmonary artery were selected, because the opening of the right cavities of the heart is less dangerous than the opening of the left cavities.

METHODS.

The preparation of the animals is similar to that already described.[1] After the thoracic cavity has been opened in the anesthe-

* Received for publication, May 20, 1914.

[1] Tuffier, T., and Carrel, A., *Jour. Exper. Med.*, 1914, xx, 3.

tized animal by a left transverse thoracotomy, the pericardium incised, and the anterior part of the heart exposed, the operation is started. The operation consists of four stages: (1) the stoppage of the circulation of the heart, (2) the opening of the pulmonary artery, (3) the performance of the intracardiac operation and the closing of the pulmonary artery, and finally (4) the reëstablishment of the circulation through the heart.

The Stoppage of the Circulation of the Heart.—This has already been performed in many different ways. We ourselves have used all known methods of stopping the circulation through the heart. Finally, we adopted the method of clamping in mass the pedicle of the heart by means of large soft-jawed forceps. The heart was not taken out of the pericardium, but the incision of the pericardium was large enough to permit of the easy introduction of one of the jaws of the forceps under the pedicle. The forceps was a Doyen, the jaws of which were covered with rubber. One of the jaws was introduced into the pericardium under the pedicle and directed from the right to the left side by the index finger of the operator. Before clamping, the position of the forceps was carefully examined. The handling of the forceps and of the pedicle was always very gentle. Care was taken not to produce any compression of the veins before the time of the clamping. It is important for the heart to be in normal condition before clamping the pedicle, a result obtained by overventilating the blood, which is easily accomplished by means of the Meltzer-Auer apparatus. It was noticed that if the heart is clamped before it is in excellent condition the interruption of the circulation is less safe than when the heart is filled with well oxygenated blood. The advantage of using the Meltzer-Auer method in this operation is that it permits of an acceleration of the oxygenation of the blood at will. When everything is ready for the operation the forceps is rapidly clamped and, without a second being wasted, the heart or the vessel is opened and the operation started.

When these precautions are taken it is possible to clamp the pedicle of the heart for as long as two and a half or three minutes without subsequent trouble. As soon as the clamp is removed the heart resumes pulsation, and after a short time the pulsations are again

normal. In no case was there any need of massaging the heart when the interruption of the circulation did not exceed two and one half minutes; this period probably allows of a considerable margin of safety and it would doubtless be possible to prolong the operation slightly without excessive danger. But two and one half minutes appear to be sufficient time for the performance on the valves of several operations.

The above technique may appear to be somewhat crude, but experience has shown that it is simpler than the separate clamping of the arterial and venous pedicles, and requires less handling of the heart, which is an important consideration. Moreover, traumatism of the anatomical structures of the pedicle is slight, on account of the large quantity of tissue which is taken up between the jaws of the forceps and which renders their action less rough.

The Opening of the Pulmonary Artery.—The pulmonary orifice is exposed by means of an incision made through the anterior wall of the artery at the level of or a little above its junction with the heart. The incision is made with sharp scissors, or is begun with sharp scissors and finished with blunt scissors. The wall of the pulmonary artery is perforated about one and one half or two centimeters above the point of insertion of the sigmoid valves and incised from above downwards with one cut of the scissors. The location and the length of the incision vary according to the purpose. When the incision is located above the anterior valve it is not prolonged below the level of the insertion of the valve, in order not to incise the sigmoid. When the incision is made further on the left side of the artery at the level of the junction of the anterior and left sigmoids of the valve it can be prolonged further on to the cardiac wall, and an incision four centimeters in length, half on the heart and half on the pulmonary artery, could be made without danger. The short incision made above the orifice is kept open by two forceps put on the edges, when the valves are sufficiently widely exposed to be cauterized. But through such an opening it is impossible to perform a suture of the valves. Hence for these cases a long incision is made at the union of the anterior and left sigmoids (figures 1 and 2), which permits a large opening of the pulmonary artery and the possibility of operating easily on the valves

themselves. It is not necessary to cut exactly at the point of insertion of the valves. If the incision is located near the point of insertion of the valves it does not produce a marked degree of insufficiency. Moreover, the left side of the arterial cone is not covered by large branches of the right coronary artery, and the section of the wall and its suture can be made without danger to the circulation.

Immediately on opening the pulmonary artery a large quantity of dark blood is expelled from the heart, and consequently the operating field must be narrowly walled off by the silk and cotton padded towels, in order to prevent the escape of the blood into the pleura.

The Performance of the Intracardiac Operation and the Closing of the Pulmonary Artery.—As soon as the incision is complete its edges are retracted by two mosquito forceps, the blood is sponged, and the valves are exposed. Air always enters the right ventricle. No special care is taken of this, since no complications due to the air emboli through the lungs were ever observed to follow. Three kinds of operation were performed: cauterization, suture and section, and suture of the valves. The cauterization of the valves was performed with the fine point of the thermocautery. The points of insertion of the valves, their free edge, or their internal surface were cauterized. The suture of the valves was made with a straight needle No. 16 and fine silk sterilized in vaselin. The left and right valves were united by one stitch (figure 3) at a distance of about two millimeters from the insertion to the arterial wall. This produced stenosis of the pulmonary orifice. Section and suture of one sigmoid valve were made in the following way: The sigmoid valves having been exposed by a long incision through the orifice, the right sigmoid was cut in its middle with the scissors, as far as the insertion to the arterial wall (figures 4 and 5). Afterwards the edges of the wound were united near the margin of the valve by a stitch made with straight needles No. 16 and silk sterilized in vaselin.

The operation completed, the pulmonary artery is sutured. In order not to lose any time a needle No. 12 with China silk No. 1 was kept ready. A minute piece of muscle had been fixed at the end of the thread, in order that no time should be spent in making a knot after the first stitch was made; the incision is closed with a con-

tinuous through and through suture. The time occupied by the incision of the pulmonary artery, the operation itself, and the suture of the artery varied between one minute and fifty seconds and three minutes.

Reëstablishment of the Circulation through the Heart.—As soon as the suture of the pulmonary artery is complete the clamp is removed. Generally during the period of the interruption of the circulation the heart is still beating feebly, but it may have stopped completely. As soon as the blood is allowed to flow from the vessel into the heart the pulsations recommence, weak at first, but very soon become quite normal. When the interruption of the circulation did not last more than two or two and one half minutes there was no need of massage; after a few minutes the heart had recovered its normal pulsations. A gauze sponge was always applied to the line of suture and a slight degree of compression made during a few minutes. When, after that time, there was still some leakage at the line of suture, one or two complementary stitches were added. Care was taken not to close the pericardium before the line of suture was absolutely without leakage. Then suture of the pericardium and of the thoracic wall is made, according to the ordinary method. The animal is dressed and taken care of as described in a previous article.[2]

EXPERIMENTAL.

The experiments were performed on ten medium sized dogs. Nine of these animals were young adult dogs in good health, and one was a dog about seven or eight years old. In three instances the operations consisted of exploration, suture, or section and suture of the sigmoid valves; in seven instances the sigmoid valves were cauterized.

Experiment 1. Exploration of the Sigmoid Valves of the Pulmonary Artery.—Black long haired dog. March 10, 1913. Etherization by the Meltzer-Auer method. Transverse left thoracotomy by the ordinary technique. Incision of the pericardium. Clamping of the pedicle of the heart with a soft-jawed forceps protected with rubber. Incision of the anterior wall of the pulmonary artery just above the sigmoid valves. The edges of the opening were retracted with two forceps. The sigmoid valves of the pulmonary artery could easily be seen and

[2] Tuffier and Carrel, *loc. cit.*

handled with the fingers or with the forceps. Then the incision of the pulmonary artery was closed by means of a continuous suture with China silk and a needle No. 12. The clamp was removed and the circulation reëstablished. The interruption lasted two minutes and fifty seconds. The pulsation of the heart started immediately without massage. After a few minutes it was normal. Closing of the pericardium and of the thoracic cavity by the ordinary method. The animal had no shock and walked about half an hour after the operation. During the afternoon it ate and drank as usual. May 20. Animal is still entirely normal.

Experiment 2. Suture of the Sigmoid Valves of the Pulmonary Artery.—Black and white long haired male setter dog. March 12, 1914. Etherization by the Meltzer-Auer method. Transverse left thoracotomy by the ordinary method and clamping of the pedicle of the heart. Section of the wall of the pulmonary artery and of the cardiac wall at the point of union of the anterior and left sigmoid valves. The valves were widely exposed by retraction of the edges of the wound by two forceps, and the right and left sigmoids were united at a distance of about 2 mm. from their insertion to the wall by a stitch made with a straight needle (Kirby No. 16). Then the edges of the incision of the pulmonary artery were sutured by the ordinary method. The clamp was removed after an interruption of the circulation lasting two minutes and twenty-five seconds. The pulsation of the heart started immediately without massage. Then the pericardium and the thoracic cavity were closed. After the operation the animal recovered, as in experiment 1. May 20, 1914. Animal is still in normal condition.

Experiment 3. Section and Suture of the Right Sigmoid Valve and of the Pulmonary Artery.—Yellow and white male fox-terrier, about seven or eight years old. March 17, 1914. Etherization by the Meltzer-Auer method. Opening of the chest by left transverse thoracotomy. Section of the pulmonary artery just above the pulmonary orifice, after the pedicle of the heart had been clamped. The sigmoid valves were exposed by retraction of the edges of the incision by two forceps. Then the scissors were introduced into the artery and the right sigmoid valve was completely sectioned in its middle. Next, the edges of the valve were approximated at the upper end of the incision by one stitch made with a needle No. 16 and fine silk sterilized in vaselin. The pulmonary artery was closed by the ordinary method and the circulation was reëstablished after an interruption of two minutes and thirty seconds. The operation was completed in the ordinary way and the animal not only sustained no shock but remained in excellent condition. May 20, 1914. Animal still is in excellent health. Slight diastolic murmur.

Experiment 4. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Young brindle female bulldog. April 14, 1914. Etherization by the Meltzer-Auer method. Transverse thoracotomy and clamping of the pedicle of the heart by the ordinary method. Incision of the pulmonary artery just above the pulmonary orifice. The edges of the incision were retracted by two forceps and the sigmoid valves widely exposed. The blood was removed with sponges and the edges of the left and right sigmoid valves were cauterized with the thermocautery. Then the wall of the pulmonary artery was closed and the circulation reëstablished after an interruption of two minutes. The operation was completed by the ordinary method. The animal sustained no shock and remained

in excellent condition, as in the preceding experiments. May 20, 1914. Animal is still in normal condition.

Experiment 5. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Brindle female mongrel. April 20, 1914. Etherization by the Meltzer-Auer method. All the details of the operation were identical with those given in experiment 4. The interruption of the circulation lasted two minutes and five seconds. After the operation the animal remained in excellent condition. May 6, 1914. Animal is normal. May 8. Animal coughs. May 12. Died.

Autopsy.—Pneumonia, pericardial adhesions. Valves normal. Pulmonary incision healed, without deposit of fibrin.

Experiment 6. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Brindle female mongrel. April 22, 1914. Etherization by the Meltzer-Auer method. Transverse thoracotomy and clamping of the pedicle of the heart by the ordinary method. Incision of the pulmonary artery just above the middle part of the anterior sigmoid valve. The incision was made as far as the base of the sigmoid sinus. Cauterization of the anterior sigmoid and of the point of insertion of the right and left sigmoids. Suture of the pulmonary artery and reëstablishment of the circulation after an interruption of two minutes and fifty seconds. Pulsation of the heart weak at first, normal after a few minutes. During the suture of the pericardium a small branch of the anterior coronary artery was wounded by the point of the needle. The hemorrhage was stopped by suture of the endocardium in front of the vessel. Hemorrhage ceased. The operation was completed in the ordinary way. The animal had no shock and in the afternoon was in excellent condition. April 28. Animal sick. April 29. Died.

Autopsy.—Wound infection; purulent pleurisy; no pericarditis, but pericardiac adhesions. Interior of heart normal; no thrombosis of pulmonary artery; thin layer of fibrin on the lower part of the line of suture; sigmoid valves normal; no deposit of fibrin at cauterized points (figure 6).

Experiment 7. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White and black mongrel. April 23, 1914. Etherization by the Meltzer-Auer method. The technique of the operation was identical with that used in experiment 6. But after the circulation had been interrupted and the pulsation of the heart had recommenced, fibrillary contractions suddenly appeared and the animal died. The fibrillary contractions were probably due to the fact that the incision had been made too low on the anterior part of the heart and that a deep stitch possibly included a branch of the coronary artery.

Experiment 8. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White female fox-terrier. April 28, 1914. Etherization by the Meltzer-Auer method. Incision of the skin at 10.10 A.M. Opening of the chest by transverse thoracotomy on the left side at 10.18. The pedicle of the heart was clamped at 10.30. The incision of the pulmonary artery, the cauterization of the valves, and the closing of the pulmonary artery were performed with the ordinary technique. The interruption of the circulation lasted one minute and fifty seconds. The operation was completed at 10.45. The animal was in excellent condition. May 20. Animal is still normal.

Experiment 9. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White and yellow male fox-terrier. April 29, 1914. Etherization by the Meltzer-

Auer method. Transverse thoracotomy. The heart was clamped for two minutes, during which time the incision of the pulmonary artery, cauterization of the valves, and suture of the arterial wall were made. The operation was completed and the dressing made. Same technique as in experiment 6. Animal was in excellent condition. May 20. Animal still normal.

Experiment 10. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White fox-terrier with brindle spots. April 30, 1914. Etherization by the Meltzer-Auer method. The technique was exactly the same as in experiment 9. At 11 A.M. incision of the skin. At 11.05 opening of the chest. At 11.09 opening of the pericardium. At 11.15 clamping of the heart, which lasted one minute and fifty seconds. During this period the pulmonary artery was cut, the valve cauterized, and the arterial wall sutured. At 11.27 the suture of the muscles was made, and at 11.32 the operation was completed. The animal was in normal condition. May 20. Animal is still normal.

RESULTS.

The results of these operations must be considered from the standpoint both of the general condition of the animals and of the modifications of the sigmoid valves of the pulmonary artery. Out of ten animals operated upon, three died and seven recovered and remained in excellent condition. The deaths were due to different causes. In experiment 5 the animal recovered completely and was apparently normal sixteen days after the operation. Then it sickened and died of pneumonia twenty days after the operation. In experiment 6 the cause of death which occurred seven days after the operation was a purulent pleurisy. In experiment 7 the animal died on the operating table of fibrillary contractions of the heart. It is probable that some of the causes of death can be eliminated. The infection of the thoracic wall and the purulent pleurisy, as well as the pneumonia which occurred in experiment 5, are preventable complications. They were caused by faulty technique which can be avoided. In all instances in which a few persons only were present in the operating room, the operation could be conducted with great care and all the animals recovered. It is probable that the proportion of deaths obtained in the first series of experiments will be much lower in the future. The general condition of the animals that survived was excellent. They sustained no shock; usually one hour after the operation they walked about, and three or four hours afterwards they ate and drank. These results were observed not only on young dogs, but also on a dog seven or eight

years old. All the animals were operated upon more than one month ago, and are at present normal.

The local modifications brought about by the operations were studied on the two specimens taken from animals 5 and 6, and also from the clinical examination of the seven surviving animals. The heart taken from animal 6 showed the result of the cauterization of the sigmoid valves of the pulmonary artery seven days after the operation. There were pericardiac adhesions on the anterior wall of the heart, but the heart itself was apparently normal. No thrombosis of the pulmonary artery existed. Above the anterior valve was the cicatrice of an incision one and one half centimeters long, which extended almost to the bottom of the sigmoid sinus (figure 6). The lower part of the incision was covered by a thin film of fibrin. In the upper part the stitches could be seen; the union of the edges of the wound was perfect. The margin of the anterior valve was irregular, owing probably to the fact that it had been cauterized. But there was no apparent deposit of fibrin. The left and right valves were normal. In experiment 5 the specimen showed the result of the operation after twenty-two days. The anterior and posterior parts of the heart were almost completely adherent to the pericardium. The cicatrice of the incision was seen just above the anterior valve near its left insertion in the anterior wall. It was entirely cicatrized, the stitches could not be seen, being covered by a smooth, glistening membrane. The margin of the right sigmoid was slightly irregular, owing probably to the cauterization. The two other sigmoids were normal. The examination of the two specimens demonstrated that it was possible to make a section of the wall of the pulmonary artery without injuring the valves, and that the cauterization of the valves does not produce thrombosis.

The condition of the heart of the seven animals that remained in good health was examined clinically. Six of the animals were entirely normal. In experiment 3 the animal presented a slight diastolic murmur. This animal had undergone a section of the right sigmoid valve and suture of the valve by one stitch placed near the margin. It is probable that the bottom of the valve did not unite and that a slight amount of insufficiency persisted.

CONCLUSION.

Incision, suture, and cauterization of the sigmoid valves of the pulmonary artery have been performed successfully in dogs. In the first series of ten animals, there were only three accidents, probably from largely preventable causes, leading to the death of the animals.

EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. Incision of the pulmonary artery at the union of the anterior and left sigmoid valves.

PLATE 4.

FIG. 2. Same as figure 1.

FIG. 3. Suture of the right and left sigmoid valves.

FIGS. 4 and 5. Section and suture of the right sigmoid valve.

PLATE 5.

FIG. 6. Specimen taken seven days after the cauterization of the sigmoid valves.

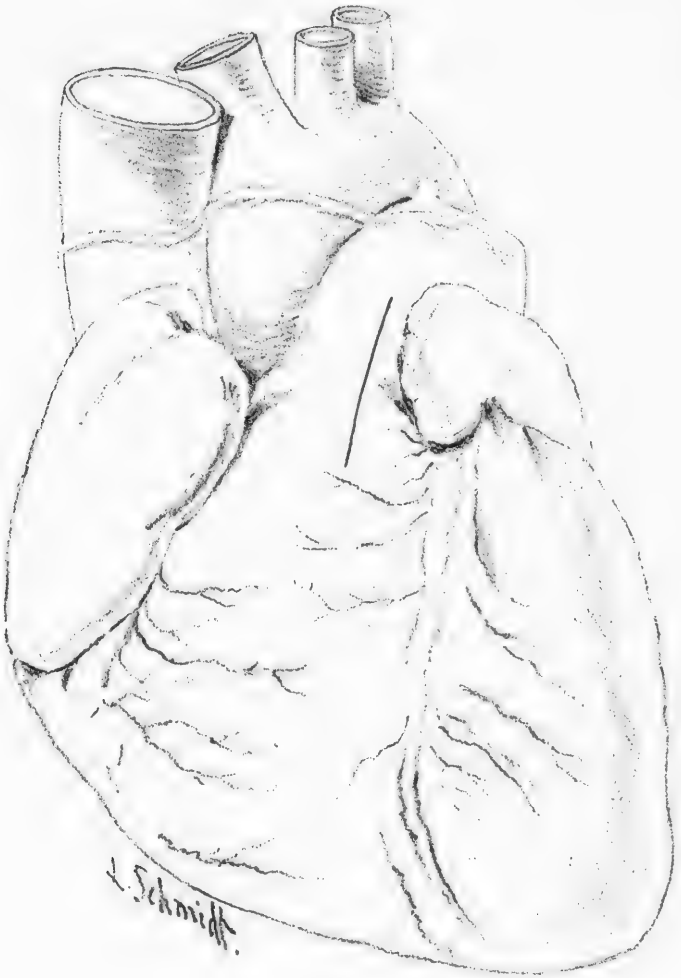

FIG. 1.

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

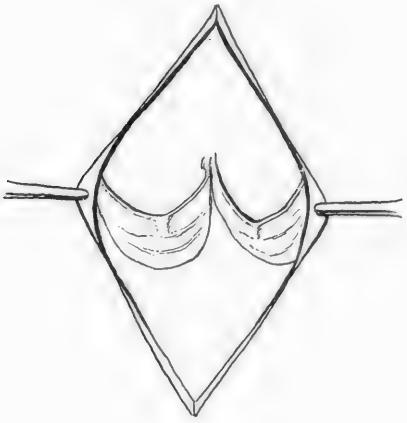

FIG. 2.

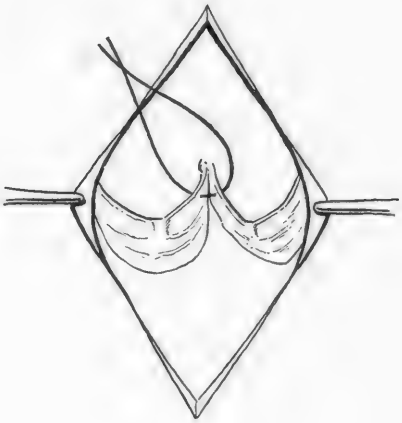

FIG. 3.

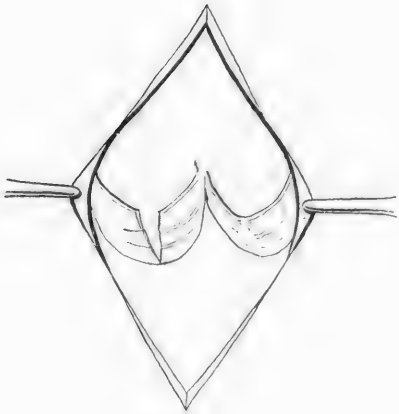

FIG. 4.

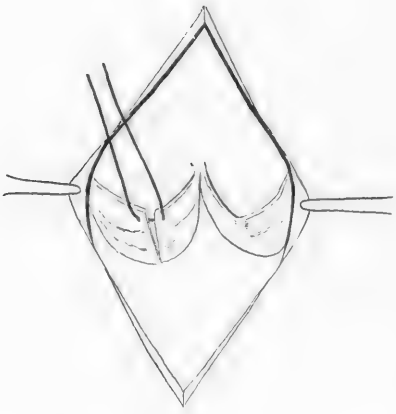

FIG. 5.

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

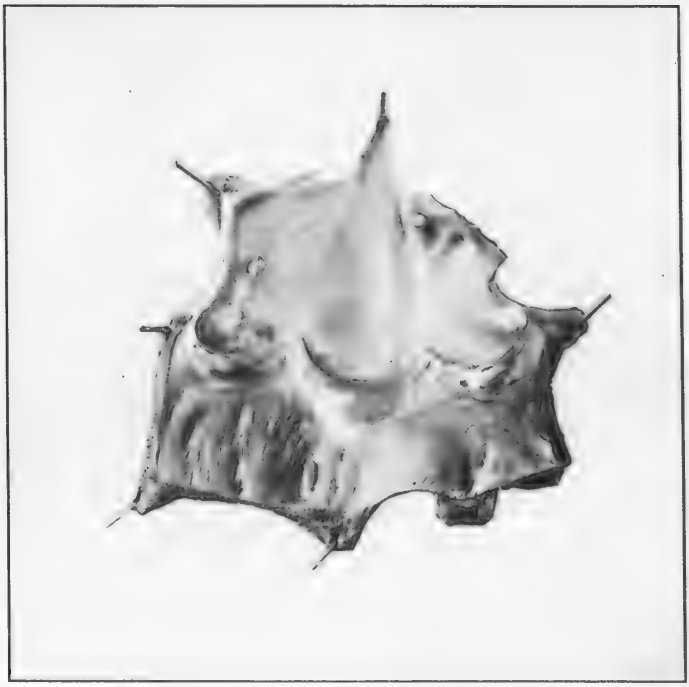

FIG. 6.

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

IX. The Changes in the Bone Marrow after Splenectomy.*

By RICHARD M. PEARCE, M.D., and O. H. PERRY PEPPER, M.D.

(*From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.*)

In connection with the various investigations[1] carried out in this laboratory on the subject of the spleen in its relation to blood destruction and regeneration, the bone marrow of splenectomized dogs has been examined with a view to determining the compensatory or other changes following the removal of the spleen. As the material now consists of marrows representing periods varying from three weeks to twenty months after splenectomy, we consider our study sufficiently comprehensive to justify a detailed report.

In the literature of the subject, the references to changes in the bone marrow following splenectomy are for the most part casual and presented but incidentally in connection with the associated changes in the lymph and hemolymph glands. In Warthin's (1) collection of the literature up to 1903 the following references occur: Tizzoni and Fileti (2) (1880) and Tizzoni (3) (1882) observed in splenectomized dogs a transformation of the fatty marrow of long bones into red marrow. Mosler (4) (1882), working likewise with dogs, concluded that following splenectomy there may be compensatory action on the part of both lymph glands and bone marrow, the latter appearing to play an important part. In one animal ten months after splenectomy the bone marrow resembled that of leukemia. This change, however, was not constant. Laudenbach (5) (1893) observed in one dog (ten to twelve years of age), with severe anemia, signs of increased blood formation in the marrow 145 days after splenectomy. Ceresole (6) (1895), on the other hand, found in splenectomized rabbits no clearly defined new formation of the marrow. Warthin (1) (1903) states that after splenectomy in the sheep and goat slight lymphoid changes in the fatty marrow occur, but he gives no histological description. Of these changes he says:

* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, March 26, 1914.

[1] *Jour. Exper. Med.*, 1912, xvi, 363, 376, 758, 769, 780; 1913, xviii, 487, 494, 665.

"The beginning lymphoid changes in the fatty bone marrow in the second and fifth month after splenectomy, are to be regarded as compensatory only for the increased destruction of red blood cells and not for any abrogated splenic function of red cell formation."

Other references may be found to changes in the bone marrow in the presence of diseases of the spleen in man (7) and in experimental anemias of animals with or without splenectomy, but few findings after simple removal of the normal spleen are available. Among the latter are Pugliese's (8) observation that after total splenectomy the bone marrow of the hedgehog becomes filled with giant cells. This change Foa (9) has found not to be characteristic of the rabbit. Vulpius (10) who in 1894 reviewed thoroughly the subject of the surgery and physiology of the spleen, and adds some experimental observations, supports the theory of increased activity of the bone marrow after splenectomy. Winogradow (11) found red marrow in the long bones of a dog 132 days after splenectomy, but yellow marrow was present in two after 517 and 760 days respectively, though one of the latter was slightly streaked with red. Hodenpyl (12) in the description of a case of absence of the spleen in man makes no mention of the bone marrow. Taylor (13) describes the marrow of two splenectomized dogs; that from an animal receiving albumoses by mouth and by hypodermic injection, and killed after nine months, was red; a second splenectomized animal, not receiving albumoses, showed a yellow marrow at the end of one year. Freiberg (14) states that he found red marrow in splenectomized animals, and Gibson (15) notes that in a dog killed five and a half months after being deprived of the spleen, the marrow was apparently in the process of change from yellow to red.

In some of these accounts brief mention is made of the increase of giant cells or of pigmented cells or of the numerical relations between the myelocytes and the white and red cells, but we have been unable to find an adequate account of the histology of the bone marrow after splenectomy based on modern conceptions of the cytology of this tissue. Histological descriptions exist, but they are either brief and fragmentary or are based on views current before the attainment of our present detailed knowledge of the morphology of the cells of the blood.

METHODS.

Our studies are based chiefly on the changes in the marrow of the long bones and particularly in that of the femur. As this marrow in the adult dog is normally fatty, objection may be raised against its use, and to overcome this objection, we attempted to study the marrow of the compact bones. The methods of decalcifying the tissues have, however, in our hands, failed to yield satisfactory

histological preparations. The alternative, the use of film preparations obtained successively at intervals over long periods of observation being impracticable, the study of cover-glass preparations was limited to a single observation at the time of the death of the animal. At the same time, however, in many instances marrow squeezed from the ribs has been obtained in sufficient amount to section and thus to allow a comparison with changes in the fatty marrow. We have, however, depended largely upon the study of sections of the marrow of the long bones and in particular of the femur. We are satisfied, as the result of our study of the marrow form a large number of normal dogs, that this is, after all, the most rational method of studying compensatory changes, for it is unusual even in a definitely fatty marrow not to find numerous centers of blood-forming cells. These may be limited to the periphery of the marrow or be scattered throughout, but whatever their position they afford an excellent starting-point for the study of increased cellular content as well as of changes in the character of the cells. The fatty marrow is of especial value in the study of the late changes, for in well fixed and well stained marrow there can be no doubt about the change from a purely fatty marrow to a red marrow rich in cells. This is so striking as to remove all doubt which exists when one examines the marrow of compact bone, as of the ribs or vertebræ, by either the section or cover-glass method.

We have worked exclusively with the marrow of the middle third of the femur, avoiding the marrow at either end, partly on account of its bony nature, but chiefly because of the occasional occurrence of more or less red marrow at the ends of the shaft. As only adult dogs have been used, we feel that the constant use of the middle portion of the marrow allows fairly comparable results. In removing the marrow half the circumference of the bone through the greater part of its length has been chipped away, and after separating the marrow from the bone and cutting it at either end it has been easily removed as a solid cylinder by gently rolling it on to a piece of filter paper. In carrying these tissues through the process of fixation and imbedding, the filter paper, which is firmly adherent to the marrow through the coagulation of the attached blood, allows the necessary manipulations without injury to the marrow

itself. The routine procedure has been to fix in Zenker's fluid without previous decalcification, imbed in paraffin, and stain with eosin and polychrome methylene blue. Other stains have, however, been used when necessary to bring out certain details.

RESULTS.

It may be stated at the outset that we have found no evidence of an early change in the bone marrow. Splenectomy does not cause, as do successive hemorrhages and hemolytic poisons, a rapid change of fatty marrow to red marrow. This latter change we have produced readily and rapidly in non-splenectomized control animals by the use of specific hemolytic serum and by causing hemorrhage, but we have never seen a frank change from yellow to completely red marrow in the ordinary course of events in the splenectomized animal until many months, usually six or more, had elapsed, and this despite the fact that many of the animals have had, as has been shown in our earlier work, a moderately severe anemia. This anemia has frequently been of as severe degree as that caused by severe successive hemorrhages in the normal dog, but changes in the marrow analogous to those caused by hemorrhage have not been evident in the earlier periods following splenectomy.

In this connection it may be recalled that the anemia of splenectomy in the dog follows a gradual downward course for three to six weeks, the decrease in hemoglobin being relatively more marked than the decrease in red cells, and that an equally gradual repair causes the red cell count and hemoglobin content to approach normal after three to four months or more. At the same time there is a transient initial leucocytosis due chiefly to polymorphonuclear leucocytes, and a more or less constant lymphocytosis with a late eosinophilia. Not infrequently the eosinophils disappear from the circulating blood from the third week until the end of the third month.

We have, therefore, in the course of our studies attempted to determine whether the hyperplasia in the bone marrow after splenectomy is compensatory in the sense of (1) overactivity in red cell formation chiefly, or (2) peculiarly active in the formation of the white cells of the blood, or (3) in the sense of an orderly reproduction of a new marrow with normal activities in the formation of all cells arising within it.

THE NORMAL MARROW OF THE FEMUR OF THE DOG.

In our study of the marrow of both normal and splenectomized animals we have used as a basis for orientation Bunting's (16) conception of erythrogenetic and leucogenetic centers, Muir's (17) descriptions of erythroblastic and leucoblastic reactions, and have received also much aid from Dickson's (18) study of the cytology of marrow. The arrangement described by Bunting is by no means a constant and definite one, but in the masses of marrow cells may be seen groups composed mainly of myeloblasts and surrounded at times by a nearer zone of myelocytes and an outer zone of leucocytes; in other groups with the same center, the outer zone may be made up of nucleated red cells with a still more distant zone of normocytes. We are not convinced that centers for the production exclusively of red cells or of white cells exist, for frequently an intermingling of the two types is seen in one center, but this conception of definite centers is of great assistance in the interpretation of marrow changes.

The study of the marrow of the femur from many normal dogs has led to our recognition of four definite groups of cells: (1) Groups of undifferentiated cells and myelocytes. These lie between fat cells and seem to be in no way connected with blood channels. In all these centers the cells of the connective tissue reticulum are in evidence. (2) Groups of the character described above, but with a peripheral accumulation of cells in which those of the leucocyte series predominate. (3) Groups as in (1), but with a mantle of cells in which those of the erythrocytic series are most in evidence. (4) Groups as in (1), but with an indiscriminate mingling of cells of red and white series.

These groups cannot always be differentiated for not infrequently an indiscriminate mingling of cells obscures the recognition of centers. Moreover, at times may be seen groups composed purely of white cells or of red cells without myelocytic centers. We have, however, found that search for the groupings described greatly facilitates the study of complex marrow pictures and leads readily to a decision as to whether leucoblastic or erythroblastic activity predominates.

In one respect the study of normal marrow has not helped us

greatly. Megakaryocytes and polykaryocytes are so infrequent in the normal fatty marrow that we have no basis, in regard to them, for a comparison with hyperplastic marrow. The same holds true for the large endothelial cells which are phagocytic for red cells and are found so frequently in hyperplastic marrow to contain remnants of red cells and fragments of pigment.

THE MARROW OF SPLENECTOMIZED ANIMALS.

In table I the general results of our observations are presented. The terms "yellow" and "red" refer to the gross appearance, not of the surface of the marrow, but of the cross section. "Slight streaking" and "streaked" refer to an intermingling of yellow and red marrow. A marrow is described as "red" only when it is uniformly so. As will be seen by a comparison of gross and microscopic appearances, a marrow "yellow" to the naked eye may, microscopically, show evidence of beginning hyperplasia. The

TABLE I.

Hyperplasia of the Marrow of the Femur after Splenectomy.

Dog No.	Period after splenectomy.	Gross appearance.	Microscopic change.
50	24 dys.	Yellow	Slight.
23	39 dys.	Yellow	None.
21	40 dys.	Yellow	Slight.
86	42 dys.	Yellow	Slight.
79	60 dys.	Yellow	Slight.
82	63 dys.	Slight streaking	Slight.
17	84 dys.	Yellow	Slight.
10	6 mos.	Red	Complete.
39	7 mos.	Red	Complete.
32	8 mos.	Yellow	Slight.
44	$9\frac{1}{2}$ mos.	Yellow	None.
41	10 mos.	Yellow	None.
24	1 yr.	Red	Complete.
59	$1\frac{1}{2}$ yrs.	Red	Complete.
57	$1\frac{1}{3}$ yrs.	Streaked	Partial.
33	$1\frac{1}{4}$ yrs.	Red	Almost complete.
51	$1\frac{5}{6}$ yrs.	Yellow	Slight.

early changes are indicated by the word "slight." The word "complete" indicates that only an occasional fat cell is seen microscopically. "Almost complete" means that fat cells are present in the proportion of one part to nine of marrow cells, in the surface area of sections studied. Several purely fatty marrows represent-

ing periods between five and twenty-four days after splenectomy are not included in the table.

The bone marrows representing the earlier periods of splenectomy, in that they show practically no changes, may be dismissed briefly. This is true of a series from animals killed at various intervals from five days to three months. Some of these marrows cannot be distinguished from those of the normal dog. In others, slight replacement of fatty tissue is seen. Thus, one representing the twenty-fourth day shows here and there between the fat cells single rows of blood-forming cells with now and then clumps of ten to thirty or more. These areas are neither purely erythrogenetic or leucogenetic, though in some of the groups with an older type of cells there is a predominance of polynucleated cells. The endothelial cells of the reticulum not infrequently contain large masses of old blood pigment.

Another, representing the fortieth day, presents practically the same appearance with a tendency, however, to greater erythrogenesis. On the other hand, a thirty-nine day dog shows a simple fatty marrow with no evidence of active blood formation. Three other marrows of this period, however, show already the early stages of hyperplasia. In one of these (forty-second day) showing a slight general hyperplasia, both types of cell groups can occasionally be isolated, but usually the groups are mixed. Greater numbers of eosinophil cells, both myelocytic and polymorphonuclear, are present than have been evident in earlier periods. A number of cells throughout the section correspond to Longcope's (19) small lymphocytes and a smaller number to Longcope's large lymphocytes. The small lymphocytes are not, however, in pure groups. The picture as a whole is more one of leucogenesis than of erythrogenesis. Very few giant cells are seen and only occasional phagocytes. Polymorphonuclear leucocytes are abundant.

In another marrow of the sixty-third day, a moderate peripheral hyperplasia of mixed type is present. Marked congestion is evident between the fat cells, and in places near the periphery there is hyperplasia; in some places the erythrocytes appear to be outside the vessel, forming distinct hemorrhages. A few phagocytes are present, but giant cells are rare. Polymorphonuclears are frequent and of

mature development. At the periphery erythrogenesis seems to predominate over leucogenesis. Eosinophils and lymphoid cells are not conspicuous.

A marrow of the sixtieth day shows less hyperplasia, but leucocytic reaction is more evident, though erythrogenesis is active. Scattered throughout the section are many small lymphocytes, but nowhere are these seen in solid clumps. Numerous deposits of pigment are seen.

Again on the eighty-fourth day an essentially fatty marrow shows a narrow cellular strip at the periphery in which erythrogenesis is active. Here and there leucogenesis predominates, but in the main the process is erythrogenetic. A few nucleated red cells of the megaloblastic type are found, but the more mature normoblasts are more abundant. In some centers radiating lines of four or five normoblasts are seen. Few giant cells are present.

The changes of the fourth and fifth months after splenectomy are not represented in this study. Well marked hyperplasia is, however, present in bone marrow representing periods of 6, 7, 12, 17, 18, and 20 months after splenectomy. On the other hand, two marrows representing respectively nine and a half and ten months show no departure from the normal fatty marrow, and in a third (eight months) only slight hyperplasia is evident. In the latter are areas composed almost entirely of cells of the myelocyte or premyelocyte type with some evidence of the formation of both red cells and polymorphonuclear leucocytes. The picture suggests a proliferation of the primitive cells of the marrow without, however, a very active function on their part. With evidence of well marked hyperplasia at six and seven months and after a year and a year and a half, it is impossible to explain its failure in these three animals representing the 8th, 9th, and 10th months respectively.

The best opportunity of studying the late changes is presented by material from six animals, representing the period from six to twenty months, in all of which the fatty marrow of the femur was transformed entirely or in large part into red marrow. The histological picture of each of these will be given in detail.

Dog 10.—Splenectomized May 20, 1913. Before operation the red cells numbered 6,910,000 and the hemoglobin was 105. The severest anemia was about

July 21; red cells 4,240,000, hemoglobin 62. On Sept. 11 the figures were 5,220,000 and 92. Later the animal became pregnant and anemia recurred, the picture on Nov. 18 being red cells 4,410,000, hemoglobin 78 per cent. On Nov. 24 the animal was chloroformed. At autopsy the medulla of both femurs presented a deep red marrow.

Histological Examination.—A uniformly cellular tissue is seen with occasionally a fat space here and there at the periphery. For the most part this marrow is as definitely cellular as is, for example, a lymph node or the spleen, and indeed it has the appearance of the pulp of the latter organ in the new born puppy. In this cellular mass, which at first appears to present a hopeless confusion of cells, it is not difficult to resolve the cells into more or less distinct proliferating centers. The arrangement is by no means a definite one, but in the patchwork of cells one sees groups which correspond to Bunting's description. In speaking of these centers we shall refer to them as erythrogenetic or leucogenetic, according to whether red cells or polymorphonuclear leucocytes predominate in the mass of cells surrounding the center in question. We have made no attempt to distinguish in these centers, which may include 6 to 10 or 20 to 30 cells, between the finely granular neutrophil myelocyte and the non-granular basophil cell from which it is supposed to arise. In these centers mitotic figures may occasionally be seen but only after prolonged search. It is also in these centers that old blood pigment, which is quite abundant in this marrow, is deposited; its deposition in the loose vascular tissue elsewhere has not been observed. The erythrocytic centers appear to be more active than the leucogenetic. This impression is based on the fact that about a mass of myelocytes composed of twelve to fifteen cells may be seen twenty-five to thirty nucleated red cells and a small number of normocytes, while about the leucogenetic centers comparatively few leucocytes are seen. The red cells in question vary in size and show changes from the megaloblast to the normocyte. It is not to be supposed that about erythrocytic centers no leucocytes occur. A few are always present; for example, among the twenty-five to thirty cells mentioned above, eight polymorphonuclear leucocytes could be clearly distinguished. Sometimes on one side of a center nucleated erythrocytes may be grouped, and on the other leucocytes with little intermingling. This suggests simultaneous formation of the two cells in one cell center. When this occurs the number of red cells is always greater than the number of leucocytes, in the proportion of 30 to 8. All through the section are lymphoid cells, usually single and of the small variety. Giant cells are frequent and a few show inclusions of polymorphonuclear leucocytes. Cells containing such inclusions have a broad homogeneous gray staining protoplasm suggesting necrosis. There is considerable pigment, but not many phagocytic endothelial cells are seen. Normoblasts are seen free in the capillaries. Smears from the marrow of the ribs show active erythrogenesis, and, on the whole, much the same cellular picture as the marrow just described. In the rib marrow a considerable number of eosinophils, chiefly polymorphonuclears, are also seen. In connection with the activity in the formation of red cells shown by the marrow, it is significant that the blood count six days before death was 4,100,000, and the hemoglobin 78 per cent. On Sept. 11, four months after splenectomy, the figures were 5,240,000 and 92. In other words, despite the hyperplasia of the bone marrow

the animal exhibited a late anemia, two and a half months after recovery from the initial anemia, following splenectomy. This may have been due to the drain occasioned by the intervening pregnancy,—an unfortunate complication from the point of view of the study of the blood. The fact remains, however, that the marrow is actively forming normal red cells. The anemia was, therefore, not due to insufficient erythrogenesis in the marrow.

Dog 39.—On Apr. 7, 1913, before splenectomy, the red cells numbered 6,528,000, and hemoglobin was 110 per cent. The lowest point of anemia was reached on June 3, the red cells numbering at that time 3,650,000; the hemoglobin was 62 per cent. By July 7 the blood picture had improved (red cells 5,080,000, hemoglobin 88 per cent.), but on Sept. 11, a late recrudescence of the anemia gave red cells 4,040,000 and hemoglobin 68 per cent. The animal was killed on Nov. 15. The bone marrow of the femur was of a definite red color. The anemia did not affect the general nutrition of the animal. On Apr. 7 the weight was 12,800 gm.; on Nov. 15 it was 13,950, and the adipose tissue was abundant.

This animal, representing practically the same period after splenectomy and the same changes in the blood, presents very much the same picture in the marrow. Of minor importance is the fact that the marrow is not so cellular, the proportion of cells to fat being in the ratio of about 3 to 2; also the myelocytic centers are not so pronounced, but in other respects the marrow is the same. Many giant cells are present but lymphoid cells are rare. The formation of red cells and leucocytes is perhaps not so rapid; that is, the numbers about any one center are not so great but, on the other hand, the activity of the marrow in connection with the former is sufficient to exclude the possibility of the bone marrow being responsible for the later development of anemia.

Dog 24.—This animal was splenectomized on Feb. 10, 1912, and was used for the injection of hemolytic immune serum on Mar. 20 and again on Apr. 7. On June 28 it had recovered from the anemia then produced (red cells 5,650,000, hemoglobin 89 per cent.), and on July 15 it was treated with sodium oleate. On Sept. 26 the red cell count was 5,780,000, and hemoglobin 90 per cent. On Feb. 19 the blood picture had improved (red cells 6,048,000, hemoglobin 110 per cent.), and at this time hemolytic serum was again injected. The animal was chloroformed on Mar. 4, 1913. The lapse of time since splenectomy was, therefore, thirteen months. At autopsy a red marrow was found.

Owing to the use of various hemolytic poisons, the bone marrow of this animal may have been influenced by other factors than the absence of the spleen. The histological picture, however, is so in accord with the marrow of simple splenectomy that, with this explanation, it is included in the series.

Histological Examination.—The marrow is uniformly solid with no fat spaces visible in any of the sections examined. It does not, however, appear to be as cellular as the marrow of dogs 10 and 39. This difference is caused by a greater congestion and distention of the blood vessels, a slight increase in the reticulum, and a lessened tendency of the myelocytic tissue to be grouped in large centers. Erythroblastic centers are very prominent and very active; leucogenetic centers, on the other hand, are made out with difficulty. Lymphoid elements are rare. Many cells of the myelocytic type are seen with coarse

basic granules and short threads in the nucleus, and with little or no protoplasm. In close relation to these are sometimes seen degenerated mitoses, but whether all these masses can be so interpreted is not clear. These degenerative changes are doubtless the result of the last injection of hemolytic serum.

Dog 59.—On July 24, 1913, this animal was splenectomized and used for the study of the progressive anemia following this procedure. On Dec. 7, 1912, the highest point of recovery was reached (red cells 5,250,000, hemoglobin 105 per cent.). Continued observation showed a slight decline to 5,200,000 red cells and 86 per cent. of hemoglobin on May 21, 1913, on which date the animal was used in an experiment with sodium oleate. From the moderate anemia caused at this time, the animal quickly recovered, the blood examination on June 9 showing 5,050,000 red cells and 86 per cent. hemoglobin, the condition slightly improving as to hemoglobin content until Nov. 18, 1913, when red cells were 5,100,000 and hemoglobin 101 per cent. The animal was chloroformed on Nov. 24. At autopsy the bone marrow of the femur was soft, succulent, and dark red in color. In connection with the general condition of this animal it is of interest to note that in the last seven months its weight increased from 10,450 to 12,580 gm., and that adipose tissue was very abundant. The administration of sodium oleate introduces a possible disturbing factor, but as this was given six months before death, it is not considered, in view of our other results, a serious matter.

Histological Examination.—The marrow shows some fat cells, the proportion of marrow cells to fat being about 10:1. Nothing new is presented. Leucogenesis and erythrogenesis proceed at about equal rate, the latter being a little more active. Mitotic figures are seen not infrequently, but the type of cell in which they occur is not always evident. Myeloblasts seem to be more abundant than usual. Giant cells are fairly abundant, but lymphoid cells are rare.

Dog 57.—On June 23, 1912, the blood of this dog contained 5,350,000 red cells per cubic millimeter, and 98 per cent. hemoglobin. On July 2 the spleen was removed. The resulting anemia reached its lowest point (red cells 2,970,000, hemoglobin 50 per cent.) on Aug. 5. On Oct. 24, two days after the blood count showed 5,240,000 red cells and 90 per cent. hemoglobin, the animal received sodium oleate intravenously; a very slight anemia (fall in hemoglobin to 62 per cent., but no change in red cells) resulted. In Jan., 1913, the red cells were 5,206,000, hemoglobin 110 per cent., and with slight variations this higher level was maintained, accompanied by an increase in body-weight, until Dec. 12, 1913, when the animal was chloroformed. At autopsy the animal was found to have a large amount of adipose tissue; the bone marrow of the femur was definitely reddish in color with faint yellowish streaks. As the sodium oleate given four months after splenectomy and fourteen months before death produced only a slight transient change, we consider that the bone marrow represents the effect of splenectomy only.

Histological Examination.—The relation of the fat to cells is about 1 : 1; otherwise nothing new is seen. The marrow is very active, leucogenesis and erythrogenesis being equally prominent. Phagocytic cells and masses of old blood pigment are quite numerous, as are also giant cells. More abundant than

in other marrows are eosinophils of the myelocytic type. Lymphoid cells are not conspicuous.

Dog 33.—This animal was splenectomized on May 14, 1912, the blood examination on the previous day showing 4,950,000 red cells and 85 per cent. hemoglobin. The anemia following splenectomy reached its lowest point on June 28 (red cells 3,550,000, hemoglobin 52 per cent.). On Sept. 20 the red cells had risen to 5,490,000 and hemoglobin to 95 per cent. In Nov., 1913, the animal passed successfully through pregnancy. In Jan., 1914, as the animal had developed mange, it was chloroformed. The blood examination on the preceding day was red cells 4,480,000, hemoglobin 70 per cent. At autopsy the bone marrow of the femur was deep red in color. It should be stated that one and two months before splenectomy the animal had received injections of hemolytic serum. From our studies of the effect of hemolytic serum in the normal dog, we do not believe that these injections, nearly two years before death, are in any way responsible for the hyperplasia of the marrow.

Histological Examination.—This marrow differs in no way from those of dogs 57 and 59 described above.

Dog 51.—The spleen was removed on May 31, 1912, and on June 26 hemolytic serum was administered. From the anemia thus produced the animal made a slow recovery, but after 200 days the blood examination showed 6,200,000 red cells and 110 per cent. hemoglobin, as compared with 6,210,000 red cells and 100 per cent. hemoglobin before splenectomy. On Mar. 26, 1914, when the animal was chloroformed, its weight was 9,750 gm. as compared with 8,270 gm. at the time of splenectomy, and 8,120 when hemolytic serum was administered. The notes made at the autopsy refer to the large amount of adipose tissue, the normal appearance of the lymph nodes, the absence of supernumerary spleens, and the presence in the long bones of a distinctly yellow fatty marrow.

Histological Examination.—The marrow shows a very slight hyperplasia with large numbers of leucocytes and deposits of blood pigment.

DISCUSSION.

In view of the slight changes seen in the bone marrow during the early periods after splenectomy, it appears that neither during the period of anemia and consequent repair nor in the period of hyperplasia of bone marrow are nucleated or other irregular forms of red cells found frequently in the peripheral blood. Careful differential counts of three dogs at regularly spaced intervals for 138 days failed to reveal in two the presence of nucleated red cells, and in one they were found only five times, the largest number seen in one count being three. In a large number of other animals in which differential counts were made at irregular intervals, changes in the red cells have been found very rarely; in one dog five weeks after splenectomy five normoblasts and two megaloblasts were

found (in 100 cells) with evidence of poikilocytosis and polychromatophilia, and a week after one normoblast and one megaloblast. These findings correspond to the first days of beginning repair, the red cells and hemoglobin having a few days before reached the lowest level observed during the experiment; hemoglobin 50 per cent., red cells 2,970,000. In another, two months after splenectomy, again at the stage of beginning repair (hemoglobin 92, red blood cells 3,650,000) five nucleated red cells were found, and a polychromatophilia was evident. In no instance did these findings persist for any length of time. If they have any significance it is that they indicate the period of beginning repair.

It is difficult to bring the changes in the bone marrow into relation with the changes in the peripheral blood. If the hyperplasia of the bone marrow is compensatory to increased blood destruction, or decreased blood formation, one would expect definite hyperplasia to be present in the earlier period, during the first three months after splenectomy, at a time when the anemia is evident and repair is taking place, and not after six months to a year or a year and a half when the blood picture is normal. It is true that in two of the animals (dogs 10 and 39) a late recrudescence of anemia occurred and the marrows of these animals were obtained during this period, but this was not the case in other animals of the series and is not characteristic of the late periods after splenectomy. It is therefore impossible, on account of the late development of hyperplasia in the marrow, to explain its occurrence as compensatory to the anemia following splenectomy.

Likewise we cannot accept Warthin's theory based upon his study of sheep and goats. In these animals Warthin found hyperplasia of the marrow to occur several months after splenectomy and to be associated with evidence of increased destruction of red blood cells in the lymph and hemolymph nodes. This destruction, greater than that in the primitive spleen, is responsible, he believes, for the anemia following splenectomy, and this is in turn compensated by increased activity in the bone marrow. We have found little to support this theory in our studies of the dog. The lymph nodes, as well as the endothelial cells of the liver, as we have shown elsewhere (20), are indeed more active after splenectomy than in the

normal animal in the phagocytosis and destruction of red cells, and this is very evident when large numbers of red cells are injured, as by the administration of a hemolytic poison; but in the ordinary course of events, after splenectomy, the lymph nodes present no evidence of excessive blood destruction. An occasional cell containing one or two red cells may be seen and small amounts of old blood pigment are occasionally demonstrable, but of excessive hemolysis there is no evidence. Microchemical tests for iron in the lymph nodes of fifteen splenectomized dogs showed a considerable amount of iron in three, slight amounts in five, and none in six. The animals examined represented periods of eleven days to twenty-two months after splenectomy. In the lymph nodes of eight normal animals similarly examined, moderate amounts of iron were found once, slight amounts three times, and in four, none. It is evident, therefore, that in the dog the iron content of the lymph nodes after splenectomy differs little from normal. The liver likewise shows no increased deposition of iron. Of fourteen livers from splenectomized dogs, four showed slight depositions of iron in the Kupffer cells, while ten showed none. At the same time the livers of six normal dogs were similarly examined; in three slight deposits of iron were found, and in three none. For this reason, and because the anemia is not persistent and progressive we cannot support the theory that the hyperplasia of the marrow is compensatory to abnormal blood destruction in the lymph nodes.

Another possible explanation is that the bone marrow, in the absence of the spleen, is concerned in the storing and utilization of iron. There is no doubt that, in the intact animal, iron set free in the dissolution of red cells is stored in the spleen. After splenectomy a readjustment in the storage of iron takes place, and there is some evidence that for a short time after the removal of the spleen iron is lost to the body. It is possibly this disturbance of iron utilization that is responsible for the early transient anemia. Our investigations[2] show, however, that this disturbance of iron utilization is transient and that after a few weeks the elimination of iron in the splenectomized animal differs in no way from the process in the normal animal. This suggests naturally that the storage of

[2] To be presented in detail in a future communication.

iron in the absence of the spleen is taken over by other tissues. As microchemical tests for iron showed no definite increase of iron in the lymph nodes and liver it seemed probable that the bone marrow might be the chief depot of iron storage. Such a view was supported by the fact that all hyperplastic bone marrows contain large amounts of altered blood pigment, sometimes free, but occurring, for the most part, in large phagocytic cells. The activity of these phagocytic cells presumably in transforming the iron of old blood pigment in order that it may be reutilized by the red cells, might, it was plausible to suppose, stimulate the other functions of the bone marrow, that is, the erythrogenetic and leucogenetic functions and cause eventually a replacement of the fatty marrow by a very cellular red marrow.

In order to prove this hypothesis it was necessary to obtain some idea of the iron content of these marrows. Direct chemical analysis was out of the question on account of the small amount of material available and the variations in blood and bone content of different marrows. We therefore made a comparative study based on the use of the microchemical reaction for iron. This demonstrated at once that all red marrows in our series have a large content of iron, and that fatty marrows contain very little or no iron. On the other hand, when the marrows of non-splenectomized dogs rendered hyperplastic by anemia or infection were examined it was found that these also had a large iron content. Thus in a group of 17 non-splenectomized dogs iron was present in the marrow in large amounts in 4, in moderate amounts in 2, in small amounts in 4, and in 7 none was found. On the other hand, in 27 splenectomized dogs, iron was present in large amounts in 10, in moderate amounts in 3, in small amounts in 4, and absent in 10.

In both groups the amount of iron was in direct proportion to the degree of hyperplasia. These observations point therefore to the conclusion that a red marrow is always rich in iron, but it is impossible to say whether the cellular hyperplasia or the iron deposition is primary. Under the circumstances, it is also impossible to conclude that the late hyperplasia of marrow following splenectomy is an attempt to conserve iron. Moreover, the irregularity of our results as shown by the failure of hyperplasia in four animals,

representing respectively the 8th, 9th, 10th, and 22d months after splenectomy, prevents in the present state of our knowledge an adequate explanation of the cause of the transformation from yellow to red marrow.

The divergent results in this study are characteristic of all phases of experimental work on the spleen and doubtless are to be explained by the fact that removing the spleen takes away only one organ of a system composed of liver, spleen, lymph nodes, and bone marrow, and that the interrelations which exist in this system may or may not under varying circumstances bring into play compensations of the greatest importance in determining the degree of blood destruction or regeneration and therefore the degree of change in the bone marrow.

A search of the literature of splenectomy in man, although it reveals evidence of the occurrence of red marrow in various forms of splenic anemia (7), offers little of importance concerning the changes which occur in the bone marrow after removal of the normal spleen. Several references (21) are made to the occurrence of pain in the long bones after splenectomy, and by some this has been assumed to be evidence of hyperplasia within the rigid bony canal. The only note of the direct examination of the bone marrow after splenectomy is that of Riegner (22), who found active proliferation of the marrow of the femur in a man whose leg was amputated for gangrene four weeks after splenectomy for trauma. It is therefore impossible, on account of this paucity of data concerning the changes in man, to bring them into relation with our experimental results.

CONCLUSIONS.

Splenectomy in the dog causes, as a rule, a transformation of the fatty marrow of the long bones to a richly cellular red marrow.

During the early periods, one to three months, the change in the marrow is slight and either focal or peripheral; after six to twenty months the replacement of fat by marrow cells is complete or nearly so. Exceptions were, however, seen in four animals representing the 8th, 9th, 10th, and 22d months, respectively. The evidence at hand does not support the theory that this hyperplasia is compen-

satory either to the anemia caused by splenectomy or to an increased hemolysis in the lymph nodes. It is possible that it may be a concomitant of the activity of the bone marrow in taking over, in the absence of the spleen, the function of storing and elaborating the iron of old blood pigment for future utilization by new red cells, but our studies do not fully support this view.

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THE MECHANISM OF ANAPHYLATOXIN FORMATION.

Studies on Ferment Action. XV.*

By JAMES W. JOBLING, M.D., and WILLIAM PETERSEN, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.)

The attention that the problems of anaphylaxis and of anaphylatoxin formation have received during the past few years has led to two antagonistic conceptions as to the nature of the mechanism of intoxication.

Friedberger (1) believes that the phenomena are due to an intoxication by protein split products, placing the matrix of the poison in the foreign protein, or antigen, introduced. Doerr (2) regards them as physical and as resulting from colloidal changes in the serum, and denies the possibility of enzymotic protein cleavage as having any direct bearing on the process of intoxication. Keysser and Wassermann (3), Ritz and Sachs (4), and Bordet (5) are more or less committed to the physical theory, although they do not consider it necessary to go as far as Doerr in denying the splitting of the proteins, merely placing the matrix of the toxin in the homologous serum, without definite agreement as to the nature of the colloidal changes that are supposed to occur.

The production of the so called anaphylatoxin *in vitro* by Friedemann (6) and by Friedberger and Mita (1), by the interaction of complement, antigen, and amboceptor, has been greatly simplified. Friedberger himself found the amboceptor superfluous. Almost any adsorbing substance has been used as an antigen; such as kaolin (Keysser and Wassermann (3)), agar (Besredka and Ströbel (7), and Bordet (5)), *"Kieselguhr"* (Doerr (2)), starch (Nathan (8)), waxes and fats from bacilli (Leschke (9)), besides the true antigens, which latter need not be specific, for they are still effective after boiling. The experiments of Doerr and Russ (2), Kraus (10), Seitz (11), Aronson (12), and Friedberger have shown that complement is not essential, for toxic sera can be produced from inactivated serum, although not with the same regularity as from active serum.

If, then, the antigen-antibody hypothesis has failed to explain the accumulated facts, how are we to interpret the toxicity of the so called anaphylatoxins? The evidence still seems strong that the

* Received for publication, April 2, 1914.

toxic substances are cleavage products of proteins, or, at any rate, proteins so altered that they are capable of being split in the organism; for Friedberger does not claim that the entire toxic effect of anaphylatoxin is due to preformed split products. The arguments made against the protein-intoxication conception, such as the time element and the minute amount of substance necessary, are not convincing, for we know that ferment action may be very rapid; and as far as the quantity of substrate is concerned the argument fails if we place the matrix of the poison in the serum proteins themselves. There is some reason to believe, however, that if ferment action is the basis of anaphylactic shock, these ferments may have a much wider range of action than merely on the introduced protein.

Doerr (2) assumes that guinea pig serum is normally toxic, but that the toxicity is held in abeyance by some unknown substance which is adsorbed during the anaphylatoxin formation. Ritz and Sachs (4) suggest that during adsorption alteration occurs in the serum resulting in the formation of toxic substances. In a measure, both of these views are probably correct, although in neither of the papers in which they are put forward are there to be found suggestions as to the nature of the adsorbed substances. Doerr calls attention to the marked changes in coagulation as manifestations of the intoxication and suggests a causal connection. We, too, feel certain that this phase warrants a more detailed study.

Granting tentatively that anaphylaxis and anaphylatoxin intoxications are due to protein split products, we come to a consideration of the matrix. That the antigen used in producing anaphylatoxin is not split, has been convincingly shown by Donati (13) who noted that organisms so used retained their antigenic properties; while Neufeld and Dold (14) observed that living bacteria produced anaphylatoxin more easily than killed organisms. The fact that the same antigen can be used repeatedly would also tend to confirm this view. The demonstration of dialyzable split products occurring in anaphylatoxin (Pfeiffer and Mita (15)) gives no clew to the origin of the cleavage products; and inasmuch as we have recently (16) shown that normal serum will autolyze rapidly when the antiferments are lowered or removed, there is reason to believe that small amounts of split products may occur in anaphylatoxin. Total non-coagulable nitrogen determinations made according to the method of Folin show, however, that whatever splitting takes place in the usual methods of anaphylatoxin formation is very small. The production of anaphylatoxins by means of kaolin, agar, starch, waxes, and infusorial earth leaves no room for doubt that the matrix must reside in the serum.

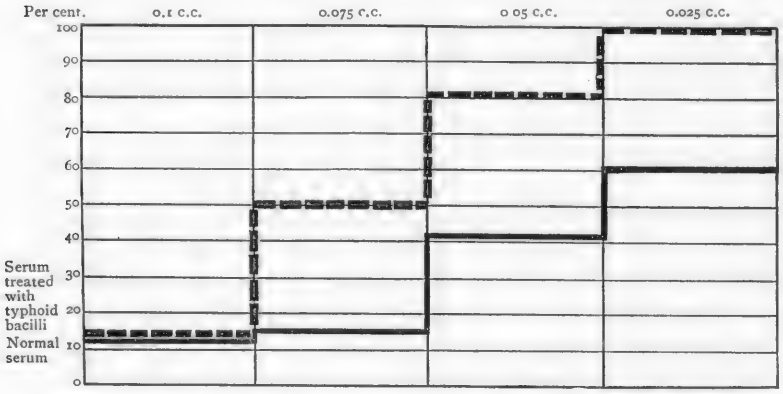
In a recent paper (16) we have demonstrated the lipoidal nature of serum antitrypsin and have shown that the inhibition is due to

unsaturated fatty acids and esters (17). We have also shown (18) that by extracting the serum with chloroform or ether, thereby removing the antitrypsin, or by saturating the free bonds with iodin, or by acidulating and filtering the serum, the serum becomes highly toxic, this toxicity depending on three factors; *i. e.*, changes in coagulation, exposure of serum proteins, and autolysis *in vitro* of the exposed proteins. Under these conditions the serum becomes from five to ten times as toxic as the ordinary anaphylatoxin, the exact minimum lethal dose depending on the amount of lipoids removed and the state of autolysis. The increase in toxicity is accompanied by a constant increase in the total non-coagulable nitrogen as determined by the Folin method, until a period of digestion is reached when the serum is no longer toxic. The mere inactivation of serum antitrypsin by heat or acid is not sufficient to secure a toxic serum, possibly because the lipoids remain in the serum and are rendered active again on reinjection. From the serotoxin which we have prepared we have isolated toxic split products, thermostabile, and lethal in doses of about 0.01 of a cubic centimeter per gram weight of guinea pig when made up to the original serum volume.

The fatty acids and their salts are easily adsorbable substances (19), and we might therefore expect that under proper quantitative conditions it would be possible to adsorb these protective lipoids from serum without affecting the other constituents. We have therefore examined the effect of the various adsorbing substances which have been found to produce typical anaphylatoxin, on the serum antitrypsin of fresh guinea pig serum and other sera. Our experiments have been made by incubating the mixture for three hours and then testing the antitryptic effects directly or after permitting the mixture to remain over night in the ice chest. In the charts illustrating these experiments the antitryptic value of the untreated serum is shown in the heavy black lines. Inasmuch as the ordinary Fuld-Gross technique for antitrypsin determination leads to serious errors in the reading, we have, in every instance, determined the total nitrogen digestion by the method of Folin, which we have described in our former papers.

EXPERIMENTS WITH TYPHOID ANAPHYLATOXIN.

Fresh guinea pig serum was mixed with typhoid bacilli in the proportion of two cubic centimeters of serum to one agar slant of bacilli and the mixture was incubated for three hours. The mixture was then placed in the ice chest over night. As will be seen from text-figure 1, there was a marked decrease in the antitryptic value of the serum so treated. There was no increase in the non-coagulable nitrogen of the serum. A portion of the mixture was



TEXT-FIG. 1. Adsorption of serum antitrypsin from guinea pig serum by typhoid bacilli.

centrifuged until it became clear, and was then injected intravenously into guinea pigs to determine its toxicity. Two cubic centimeters caused immediate death of a guinea pig weighing 210 grams, with all the evidences of anaphylaxis. The experiments have been repeated several times with similar results.

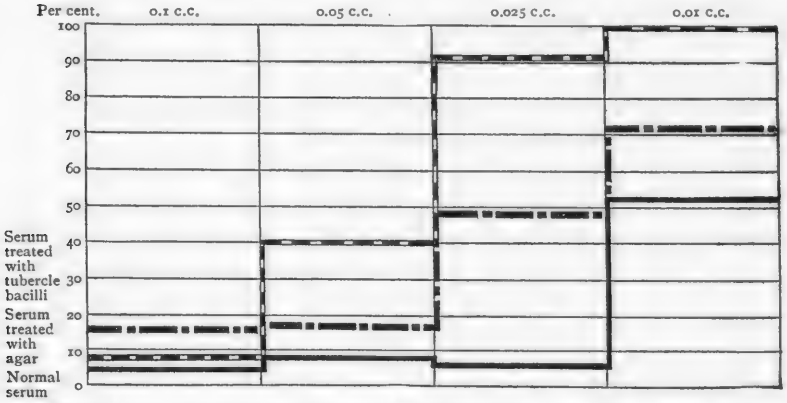
EXPERIMENT WITH AGAR AND TUBERCLE BACILLI ANAPHYLATOXIN.

A similar experiment was made with dried tubercle bacilli and also with a small amount of solid agar. As will be seen from text-figure 2, tubercle bacilli have somewhat, and the agar has greatly lessened the serum antitrypsin. It is interesting to recall that the production of anaphylatoxin from tubercle bacilli has always been somewhat difficult, and that Shibayama (20) found

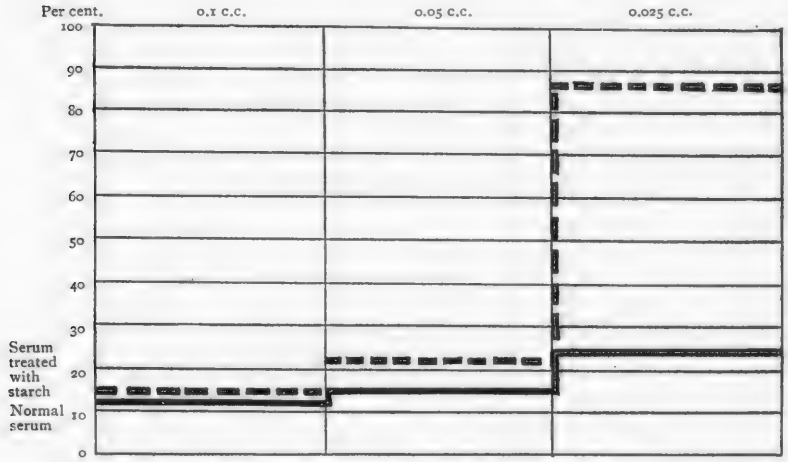
that by extracting the fats from the bacilli the result was much more constant.

ANAPHYLATOXIN FROM STARCH.

The next experiment was made with starch as an adsorbent. The data from which text-figure 3 has been made will be found in protocol I. In this experiment a large amount of starch paste



TEXT-FIG. 2. Adsorption of serum antitrypsin by agar and tubercle bacilli.



TEXT-FIG. 3. Adsorption of serum antitrypsin by starch.

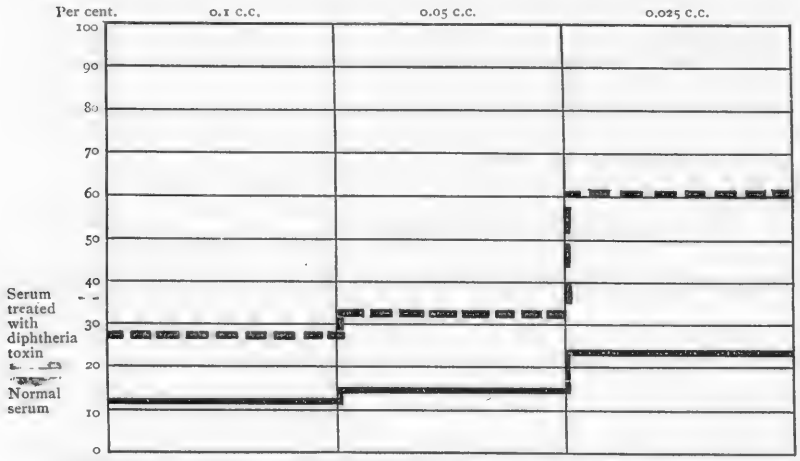
adsorbed less of the antitrypsin than a smaller amount, which is in accord with the actual toxic effects observed by Nathan (8).

The following experiment illustrates the method that we have used in this series of experiments.

2 c.c. of fresh guinea pig serum were mixed with each of the following substances:

1 c.c. of diphtheria toxin (minimum lethal dose = 0.0025 c.c.).
0.1 c.c. of diphtheria toxin (minimum lethal dose = 0.0025 c.c.).
0.2 gm. of kaolin.
0.05 gm. of kaolin.
0.1 c.c. of starch paste.
0.05 c.c. of starch paste.
0.25 gm. of agar (semisolid) active serum.
0.25 gm. of agar (semisolid) inactivated serum.

The mixtures were incubated for three hours, placed in the ice chest over night, and the next morning each tube was diluted one tenth to twenty cubic centimeters with normal salt solution. The



TEXT-FIG. 4. Effect of diphtheria toxin on serum antitrypsin.

kaolin, starch, and agar sera were then centrifuged, and the supernatant serum was mixed with trypsin in the proportion shown in protocol I, incubated for thirty minutes, and two cubic centimeters of 1 per cent. casein solution were then added. After digestion was complete, the undigested casein and serum proteins were pre-

PROTOCOL I.

No. of tube.	1 per cent. casein solution.	Trypsin.	Serum 1:10 + diphtheria toxin.		Serum 1:10 + kaolin.		Serum 1:10 + starch.		Serum 1:10 + agar 0.25 gm.		Normal guinea pig serum 1:10.	Total nitrogen digestion.	Digestion.	
			1 c.c. toxin.	0.1 c.c. toxin.	0.2 gm. kaolin.	0.05 gm. kaolin.	0.1 c.c. starch.	0.05 c.c. starch.	Active.	Inactive 54° C.				
1	2 c.c.	0.0 c.c.	.....	.....	.....	.....	.....	.....	.....	.....	.....	Trace	0%	
2	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	.....	.....	.....	1.66 mg.	100%	
3	2 c.c.	0.2 c.c.	1.0 c.c.	.....	.....	.....	.....	.....	.....	.....	.....	0.45 mg.	27%	Diphtheria toxin.
4	2 c.c.	0.2 c.c.	0.5 c.c.	.....	.....	.....	.....	.....	.....	.....	.....	0.55 mg.	37%	
5	2 c.c.	0.2 c.c.	0.25 c.c.	.....	.....	.....	.....	.....	.....	.....	.....	1.0 mg.	60%	
6	2 c.c.	0.2 c.c.	.....	1.0 c.c.	.....	.....	.....	.....	.....	.....	.....	0.26 mg.	15%	
7	2 c.c.	0.2 c.c.	.....	0.5 c.c.	.....	.....	.....	.....	.....	.....	.....	0.3 mg.	17%	
8	2 c.c.	0.2 c.c.	.....	0.25 c.c.	.....	.....	.....	.....	.....	.....	.....	0.8 mg.	47%	
9	2 c.c.	0.2 c.c.	.....	.....	1.0 c.c.	.....	.....	.....	.....	.....	.....	0.2 mg.	12%	Kaolin.
10	2 c.c.	0.2 c.c.	.....	.....	0.5 c.c.	.....	.....	.....	.....	.....	.....	0.2 mg.	12%	
11	2 c.c.	0.2 c.c.	.....	.....	0.25 c.c.	.....	.....	.....	.....	.....	.....	0.3 mg.	17%	
12	2 c.c.	0.2 c.c.	.....	.....	.....	1.0 c.c.	.....	.....	.....	.....	.....	0.3 mg.	17%	
13	2 c.c.	0.2 c.c.	.....	.....	.....	0.5 c.c.	.....	.....	.....	.....	.....	0.74 mg.	44%	
14	2 c.c.	0.2 c.c.	.....	.....	.....	0.25 c.c.	.....	.....	.....	.....	.....	0.93 mg.	56%	
15	2 c.c.	0.2 c.c.	.....	.....	.....	.....	1.0 c.c.	.....	.....	.....	.....	0.13 mg.	8%	Starch.
16	2 c.c.	0.2 c.c.	.....	.....	.....	.....	0.5 c.c.	.....	.....	.....	.....	0.48 mg.	28%	
17	2 c.c.	0.2 c.c.	.....	.....	.....	.....	0.25 c.c.	.....	.....	.....	.....	0.7 mg.	42%	
18	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	1.0 c.c.	.....	.....	.....	0.26 mg.	15%	
19	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	0.5 c.c.	.....	.....	.....	0.36 mg.	21%	
20	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	0.25 c.c.	.....	.....	.....	1.43 mg.	86%	
21	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	1.0 c.c.	.....	.....	Lost	Lost	Agar.
22	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	0.5 c.c.	.....	.....	0.35 mg.	20%	
23	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	0.25 c.c.	.....	.....	0.83 mg.	50%	
24	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	.....	1.0 c.c.	.....	0.25 mg.	15%	
25	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	.....	0.5 c.c.	.....	0.37 mg.	21%	
26	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	.....	0.25 c.c.	.....	1.25 mg.	75%	
27	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	.....	.....	1.0 c.c.	0.21 mg.	12%	Normal.
28	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	.....	.....	0.5 c.c.	0.24 mg.	15%	
29	2 c.c.	0.2 c.c.	.....	.....	.....	.....	.....	.....	.....	.....	0.25 c.c.	0.4 mg.	24%	
30	0 c.c.	0.0 c.c.	0.05 c.c. diphtheria toxin		.....	.....	.....	.....	.....	.....	.....	0.1 mg.		

All tubes were made up to 5 c.c. with normal saline solution.

cipitated by salt and acid, boiled, filtered through kaolinized hard paper filters, and nitrogen determinations made on the filtrate. The nitrogen determined for each tube in reference to the total digestion of the casein gives the exact percentage of digestion.

Text-figure 4 shows the effect of diphtheria toxin on the serum antitrypsin and illustrates the digestion in tubes 3, 4, and 5 of protocol I. The amount of non-coagulable nitrogen in 0.05 of a cubic centimeter of diphtheria toxin (the largest amount used) was found to be 0.1 of a milligram.

It will be noted that kaolin had not affected the antitrypsin when the larger amount was used, but the smaller amount (0.05 of a gram) had adsorbed a large amount. Our experience has been that agar and starch and bacteria are more effective, as a rule, than kaolin, which is only effective in a certain definite quantitative relation. In the series with agar the inactive serum also shows adsorption of the antitrypsin. We have found it on the whole more difficult to secure adsorption from the inactivated serum than from the active, corresponding to the condition which obtains with chloroform extraction. It has been demonstrated repeatedly, however, that even inactivated sera will produce anaphylatoxins although not so constantly as active sera. The relation of the toxicity of normal guinea pig serum to its antiferment content has already been discussed in a previous paper in which it was shown that the toxicity is proportionate to the degree of removal of the antitrypsin, the serum becoming more toxic with decreasing antitryptic activity. In our present work with bacteria, agar, starch, etc., we have found that the same conditions apply, the agents which absorb most antitrypsin producing the most toxic sera. For these reasons we shall not describe the numerous experiments made in the course of the work.

EFFECT OF TOXINS ON SERUM ANTITRYPSIN.

When we consider the effect produced by true toxins we must, of course, seek a different explanation than one of mere adsorption. Friedberger and Mita (21) demonstrated that an active toxin could be produced by incubating tetanus toxin, diphtheria toxin, and cobra venom with fresh guinea pig complement. Their efforts met

with least success with the cobra venom preparation. They interpreted their results as indicating that the complement had split the toxins to still more toxic products which killed with typical anaphylactic symptoms. In view of the exhaustive work that has been done in the differentiation of the toxins and their components this view as to the unity of toxin action seems at least unreasonable. It has been demonstrated that the toxins have a special affinity for lipoidal substances (de Waele (22), Raubitschek (23)). Whether this depends on the presence of unsaturated fatty acids has not been determined and we are now conducting experiments in this direction. If, then, these lipoidal bonds are first saturated by a toxin we should expect that the trypsin subsequently added would be bound to a lessened degree, since the lipoids are the same in both cases. It is, of course, quite probable that the phenomenon is a purely physical one, but it is interesting to note in this connection the close parallelism shown by the behavior of the toxins and the enzyme in their relation to these lipoids. In protocol I the effect of diphtheria toxin has already been noted.

PROTOCOL II.

No. of tube.	1 per cent. casein solution.	Trypsin.	Serum normal 1 : 10.	Serum 1 : 10 + cobra venom.	Total nitrogen digestion.	Digestion.	
1	2 c.c.	0.0 c.c.			0.0 mg.	0%	
2	2 c.c.	0.2 c.c.			1.65 mg.	100%	
3	2 c.c.	0.2 c.c.	1.0 c.c.		0.28 mg.	17%	Normal.
4	2 c.c.	0.2 c.c.	0.75 c.c.		0.41 mg.	25%	
5	2 c.c.	0.2 c.c.	0.5 c.c.		0.71 mg.	43%	
6	2 c.c.	0.2 c.c.	0.25 c.c.		1.11 mg.	67%	
7	2 c.c.	0.2 c.c.	0.1 c.c.		1.2 mg.	72%	
8	2 c.c.	0.2 c.c.		1.0 c.c.	0.31 mg.	18%	Cobra venom.
9	2 c.c.	0.2 c.c.		0.75 c.c.	0.6 mg.	36%	
10	2 c.c.	0.2 c.c.		0.5 c.c.	0.83 mg.	50%	
11	2 c.c.	0.2 c.c.		0.25 c.c.	1.2 mg.	72%	
12	2 c.c.	0.2 c.c.		0.1 c.c.	1.5 mg.	0.67%	

All tubes were made up to 5 c.c. with normal saline solution.

In our next experiment (protocol II) the results showed that cobra venom[1] also lowered the antitryptic value only to a small extent, a result to be expected from the experiments of Friedberger

[1] The cobra venom was kindly furnished us by Dr. Noguchi.

and Ito, who found it difficult to secure toxic serum in this way, having only one acute death in their series.

The reason why quantitative differences should so markedly influence anaphylatoxin formation is not clear, although we have noted frequently that large amounts of bacteria, kaolin, or starch will adsorb very little of the antitrypsin, while smaller amounts will be effective. That such a condition should be true for bacteria might seem reasonable, for when large numbers are incubated a proportion may die and undergo solution, due to the lipolytic effect of the serum, while the bacterial antiferments, together with the antiferments previously adsorbed from the serum will be thrown back into solution, rendering it more antitryptic than before. That lessened toxicity when larger amounts of bacteria are used should be due to complete protein splitting to atoxic products is out of the question, for even with a concentrated leucoprotease solution Jobling and Strouse (24) found that it required several days to secure highly toxic products from typhoid bacilli.

To show the effects of various amounts of bacteria when incubated with fresh serum, we have carried out the experiment shown in protocol III. The technique was the same as before, with the exception that only one cubic centimeter of serum was used for each mixture, the final dilution being made to ten cubic centimeters, instead of twenty. The number of bacteria used is, of course, not absolute, the measure being a large platinum loop, the amounts used being 4, 2, 1, and ½ loops to each cubic centimeter. From the results it will be seen that the staphylococci are effective only in the larger amounts, while typhoid bacilli give the most marked effects in the one and two loop mixtures, the four loop mixture being less effective.

If serum antitrypsin is adsorbed by the bacteria used in the preparation of anaphylatoxin we should expect that such bacteria would become more resistant to tryptic digestion than untreated bacteria. In order to determine this point the following experiment was carried out.

Typhoid bacilli which had been incubated with fresh guinea pig serum (complement) in the preparation of anaphylatoxin were centrifuged, washed, and dried by grinding in an agate mortar. The dry weight was 30 mg. A similar amount

PROTOCOL III.

No. of tube.	1 per cent. casein solution.	Trypsin.	Serum 1:10 + staphylococci. Loops.				Serum + typhoid. Loops.				Normal serum 1:10.	Total nitrogen digestion.	Digestion.	
			4	2	1	½	4	2	1	½				
1	2 c.c.	0.0 c.c.										Trace	0%	
2	2 c.c.	0.2 c.c.										1.54 mg.	100%	
3	2 c.c.	0.2 c.c.									1.0 c.c.	0.15 mg.	10%	Normal.
4	2 c.c.	0.2 c.c.									0.5 c.c.	0.41 mg.	26%	
5	2 c.c.	0.2 c.c.									0.25 c.c.	0.8 mg.	52%	
6	2 c.c.	0.2 c.c.	1.0 c.c.									0.35 mg.	22%	Staphylococci.
7	2 c.c.	0.2 c.c.	0.5 c.c.									0.72 mg.	47%	
8	2 c.c.	0.2 c.c.	0.25 c.c.									1.3 mg.	84%	
9	2 c.c.	0.2 c.c.		1.0 c.c.								0.26 mg.	16%	
10	2 c.c.	0.2 c.c.		0.5 c.c.								0.32 mg.	21%	
11	2 c.c.	0.2 c.c.		0.25 c.c.								0.85 mg.	55%	
12	2 c.c.	0.2 c.c.			1.0 c.c.							0.22 mg.	14%	
13	2 c.c.	0.2 c.c.			0.5 c.c.							0.48 mg.	31%	
14	2 c.c.	0.2 c.c.			0.25 c.c.							1.4 mg.	90%	
15	2 c.c.	0.2 c.c.				1.0 c.c.						0.3 mg.	20%	
16	2 c.c.	0.2 c.c.				0.5 c.c.						0.35 mg.	22%	
17	2 c.c.	0.2 c.c.				0.25 c.c.						1.4 mg.	90%	
18	2 c.c.	0.2 c.c.					1.0 c.c.					0.35 mg.	22%	Typhoid.
19	2 c.c.	0.2 c.c.					0.5 c.c.					0.62 mg.	40%	
20	2 c.c.	0.2 c.c.					0.25 c.c.					0.83 mg.	54%	
21	2 c.c.	0.2 c.c.						1.0 c.c.				0.43 mg.	26%	
22	2 c.c.	0.2 c.c.						0.5 c.c.				0.47 mg.	30%	
23	2 c.c.	0.2 c.c.						0.25 c.c.				1.1 mg.	71%	
24	2 c.c.	0.2 c.c.							1.0 c.c.			0.5 mg.	32%	
25	2 c.c.	0.2 c.c.							0.5 c.c.			0.4 mg.	25%	
26	2 c.c.	0.2 c.c.							0.25 c.c.			0.9 mg.	58%	
27	2 c.c.	0.2 c.c.								1.0 c.c.		0.31 mg.	20%	
28	2 c.c.	0.2 c.c.								0.5 c.c.		0.45 mg.	27%	
29	2 c.c.	0.2 c.c.								0.25 c.c.		0.55 mg.	36%	

All tubes were made up to 5 c.c. with normal saline solution.

of dried untreated typhoid bacilli was used as a control. They were each suspended in 3 c.c. of normal salt solution and 10 mg. of sodium carbonate, and 0.1 c.c. of trypsin solution was added to each. The trypsin was of such strength that 0.1 c.c. would digest 2 c.c. of a 1 per cent. casein solution in two hours. The tubes were kept in the incubator over night. The next morning the mixtures were acidified, boiled, filtered through kaolinized hard filters, and nitrogen determinations (Folin) were made to determine the amount of digestion.

No. of tube.	Original typhoid.	Serum-treated typhoid.	Total nitrogen digestion.	Per cent. digestion.
1	3 c.c.	0 c.c.	1.5 mg.	50
2	0 c.c.	3 c.c.	0.375 mg.	12
3	3 c.c.	Total nitrogen = 3 mg.		

The digestion of the untreated bacteria is, therefore, over four times as great as of those bacteria which have adsorbed antitrypsin from the serum.

With staphylococci this difference is not so great, but in the experiments which we have made, the digestion of the untreated bacteria is always somewhat greater than of those which have been treated with fresh serum. We have stated that one can adsorb the antitrypsin from the inactivated serum almost as well as from the active guinea pig serum, although the process seems to be slower. That even inactivated sera will form anaphylatoxins has been shown before (2, 10, 11, 12). From human serum it is much more difficult to remove the antiferment by adsorption. Horse serum antitrypsin we have found almost as easily adsorbable as the guinea pig antitrypsin.

DISCUSSION.

The interpretation of our results is, we believe, simple. The serum proteins are normally prevented from digestion intravitally by the presence of protective lipoids,—unsaturated fatty acids, but when these are removed the proteolytic ferment of the serum can rapidly split the proteins; the exposure of the proteins by partial removal of the antitrypsin through adsorption, or complete removal by means of the lipoidal solvents renders the serum highly toxic to the homologous animal. The recent work of Dold and his coworkers (25) affords a complete confirmation of this view. They state that anaphylatoxins could not be obtained *in vivo* or *in vitro*

with bacteria which had been treated with oils, with bile, or with cholesterol, but that the bacteria had not lost their power to act as antigens, and still contained active endotoxins.

Bacteria treated in this manner have, of course, been saturated with lipoids and can no longer take up more when placed in contact with fresh serum. If the bacteria had been split, as Friedberger contends, then it would be impossible to secure toxic effects *in vivo*, and their antigenic properties would be destroyed. We have repeated the experiments of Dold and his co-workers and have never noted any adsorption of antitrypsin from fresh guinea pig serum by bacteria so treated; neither did the serum so treated become toxic.

The views of Friedberger are, therefore, untenable in that they would make one uniform toxic agent responsible for the manifold toxic effects observed in disease. While split products of proteins (primary proteoses) whether from a foreign source or from the organism's own proteins, are no doubt the causative agents in many instances, the mechanism of their production is unquestionably different from the one supposed by Friedberger. From the complexity of the protein molecule, we should expect that even the higher cleavage products would show differences in their toxic, as they do in their antigenic properties; and the unfailing uniformity of the toxic effects of the anaphylatoxins, together with the lack of antigenic properties, is one of the strongest arguments that the substrate from which the toxic substances are split is the same in every case. That Friedberger (26) himself seems to realize that the position which he has taken is untenable is indicated in his recent work with kaolin, the toxic effects of which he found could be prevented by previous treatment with serum. He says: "The toxicity of kaolin is not due to mechanical injury; it is probably due to the adsorption of certain substances from cells fundamental to the life of the organism."

CONCLUSION.

1. The unsaturated lipoids (serum antitrypsin) can be adsorbed from guinea pig serum, rabbit serum, and horse serum by kaolin, starch, agar, and bacteria.

2. Diphtheria toxin and cobra venom also reduce the serum antitrypsin, possibly because of their affinity for lipoids.

3. Anaphylatoxins represent sera rendered toxic by partial removal of serum antitrypsin.

4. The matrix of the protein split products lies in the serum proteins so exposed.

5. The amount of removal of serum antitrypsin depends on definite quantitative relations; very large amounts and very small amounts of adsorbing substances are least effective (kaolin, starch, and bacteria).

6. Bacteria previously treated with serum or with oils do not adsorb serum antitrypsin.

7. Bacteria treated with serum become more resistant to the action of trypsin.

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COMPLEMENT FIXATION TESTS IN CHRONIC INFECTIVE DEFORMING ARTHRITIS AND ARTHRITIS DEFORMANS.*

By T. W. HASTINGS, M.D.

(From the Department of Clinical Pathology of Cornell University Medical College, and The Second Medical Division of Bellevue Hospital, New York.)

INTRODUCTION.

Recent studies on complement fixation with bacterial antigens have shown that there are distinct possibilities in the method for etiological diagnosis. Koessler (1) and Kolmer (2) applied it in scarlet fever, determining complement fixation with other immune reactions in horses and rabbits immunized with streptococci from scarlet fever patients. In 1904 Besredka (3) applied the test to horses immunized with streptococci. Swift and Thro (4) applied the complement fixation method in attempting to differentiate various forms of streptococci. Smith and Brooks (5) studied this immune body reaction, as well as precipitins, agglutinins, opsonins, and bacteriolysins in rabbits injected with typhoid vaccine, and found that the complement-fixing substance is produced at about the same time and in about the same ratio as other immune bodies. The test has been applied in cholera, glanders in horses, echinococcus invasion, lepra, rabies, and helminthiasis.

The publications of Schwartz and McNeil (6) suggested the possibility of detecting gonococcus infections with secondary joint invasion; and the favorable results with gonococcus vaccine in gonococcus arthritis suggested a possible successful therapy for certain cases. The article by Swift and Thro (4) on the study of streptococci with the complement fixation reaction led us to the employment of this test for bacterial infections other than gonococcal.

If arthritis deformans is an infectious disease, proof of such infection may be searched for as follows:

(1) By making cultures from the exudate in the joints and in tissues about the joints. This method has been followed unsuccessfully by many. The reason is not hard to find if one bears in mind that hematogenous infection of the joint tissues begins in the terminal end bulbs of the blood vessels and in lymph spaces, and

* Received for publication, April 20, 1914.

that it is only when the process invades the joint fluid that bacteriological examinations are positive.

(2) By making cultures from possible foci, evidenced by clinical manifestations, other than the joints.

(3) By making blood cultures for bacteriemia.

(4) By making blood tests for immune bodies against exogenous and autogenous antigens, obtained by (1), (2), or (3). The complement fixation substance (*fixateur* of Besredka) is the most appropriate for this purpose.

It must be borne in mind that (2) and (3) do not locate the infection in or about the joints. Whether or not (3) positively excludes (4) has not been determined, although theoretically this would seem probable. The relation of (4) to acuteness of infection has not been determined, excepting for the gonococcus infection by Schwartz and McNeil. The results in syphilis are not considered, since the Wassermann reaction is not a true complement fixation.

Our results with *Streptococcus viridans* tend to show the findings of Schwartz and McNeil for gonococcus infections; namely, that subacute and chronic processes give positive fixation tests, while the acute processes give negative tests. Some of our control cases of acute sepsis with bacteriemia and subacute and chronic infective endocarditis with bacteriemia gave negative reactions.

Does the complement fixation reaction indicate that the joint lesion is due to the organism giving a positive test? This may not necessarily be so, yet in syphilis the Wassermann test is not positive until the invasion has become systemic, and, as Schwartz and McNeil have shown, positive tests for gonococcus are not obtained until the infection has passed beyond the primary focus. Besredka (3) found that *fixateur* for streptococci of the pyogenic and hemolytic types is readily produced by intravenous injections, and rarely or not at all by subcutaneous inoculations of streptococci in rabbits. That the positive fixation test in infective deforming arthritis indicates that the organism used as antigen is the agent of infection, is borne out (1) by giving small doses of vaccine of the organism, producing thereby focal reactions in and about the joints; (2) by marked improvement when a vaccine of the organism fixed by the patient's serum is given therapeutically over a long period of time;

(3) by finding a primary focus of infection, *i. e.*, infected teeth, prostate, and demonstrating that autogenous antigens, prepared with cultures from an alveolar abscess, yield positive complement fixation tests with the patient's serum and, injected as vaccines in small doses, cause focal reactions.

In other instances cultures of streptococci and staphylococci from the tonsils, endometrium, and sputum have given no reactions with the patient's blood, while positive reactions were obtained with gonococci and *S. viridans* from other sources, and the patients improved under treatment without removal of the tonsils and with the persistence of the endometritis.

BACTERIA ISOLATED AND ANTIGENS USED.

Table I gives the strains of *S. viridans* isolated, their source, the diagnosis, the duration of the disease, and, in the cases of arthritis, the joint involvement. The strains are numbered consecutively as antigens for reference in the records of the complement fixation tests.

Table II gives the antigens and strains of bacteria, other than *S. viridans,* made use of for control complement fixation tests. These have been lettered from A to O.

METHODS EMPLOYED.

Antigens were prepared by suspending a cultural growth in 0.8 per cent. sodium chloride solution. The nature and source of the cultures are shown in tables I and II. Cultures were grown on plain agar, glycerin agar, North's medium (7), and in plain broth. The first generations of *S. viridans* required from two to ten days for growth, and after a few transplantations a suitable growth was obtained within forty-eight hours. The surface growth of from four to eight tubes of solid media was washed off with 0.8 per cent. sterile sodium chloride solution (2 c.c. per tube), shaken thoroughly, and killed by heating to 60° C. for thirty minutes. No advantage was obtained by shaking the suspension from twenty-four to forty-eight hours in an apparatus run by water motor, rather than by thorough shaking by hand. The suspensions were standardized by the method described (1) by Callison (8) by diluting the bacterial suspension with 0.8 per cent. sodium chloride solution, to which had been added carbol-fuchsin, and counting with the Thoma-Zeiss counting chamber, and (2) by Wright's method. The latter method was found to be the most satisfactory, and with an organism as small as *S. viridans* the clearest preparations were obtained by fixing slides with the saturated solution of bichloride of mercury for ten minutes and staining in Loeffler's methylene blue for three minutes.

TABLE I.

Strains of Streptococcus viridans Used as Antigens.

Antigen.	Laboratory No.	Diagnosis.	Source.	Duration.	Involvement.
1	43358	Polyarthritis, chronic	Prostate	15 yrs.; began in left ankle, fingers, and wrists; prostatitis for 15 yrs.	All joints except spine; periostitis; periarthritis; tenosynovitis.
2	46480 46627	Polyarthritis, acute	Alveolar abscess; jaw	9 mos.; began in knees after exposure; rheumatic throat in 1908; abscess of left lower maxilla under last molar in 1912	All joints, cervical spine; periarthritis; tenosynovitis; neuritis; phlebitis, left saphenous and popliteal.
3	48707 48556	Polyarthritis, chronic	Tooth socket, immediately after extraction (2 cultures)	12 yrs.; followed puerperium, 1 mo. after parturition; began in small joints of hands; persistent endometritis	All joints, cervical spine; periarthritis; tenosynovitis; neuritis.
4	50368	Multiple adenitis, chronic	Tooth socket, immediately after extraction, alveolar abscess	14 mos.: adenitis, left cervical, axillary, and mediastinal; excessive enlargement, diagnosed Hodgkin's disease; pyorrhea; anemia; indigestion $4\frac{1}{2}$ yrs.	
5	48970	Polyarthritis, chronic; bronchitis	Sputum	2 yrs.; began in wrists, ankles, and knees	All joints except hips.
6	49347	Tonsillitis, acute	Tonsil	3 dys.; tonsillitis	
7	49384	Tonsillitis, acute	Tonsil	2 wks.; tonsillitis	
8	41979	Infective endocarditis, acute	Blood	5 mos.; acute and chronic endocarditis	
9	52475	Still's disease, 2 yrs.	Tooth socket, immediately after extraction, alveolar abscess	2 yrs.	All joints except spine.
10	51193 51194	Arthritis, monarticular, acute; jaw	Tooth socket, immediately after extraction (2 cultures)	1 yr.; tooth removed after X-ray for apical abscess; later mild polyarthritis	Left temperomaxillary.
11	51292	Arthritis deformans, polyarticular	Tooth socket, immediately after extraction (2 cultures)	30 yrs.; began in fingers, elbows, and knees	All joints; periarthritis; tenosynovitis; neuritis.

TABLE I.—*Continued.*

Antigen.	Laboratory No.	Diagnosis.	Source.	Duration.	Involvement.
12	51189	Septicemia	Blood and spleen, post-mortem		
13	51051	Endocarditis, acute	Blood	2 mos.; endocarditis, acute and chronic	
14	53377	Spondylitis, chronic	Alveolar abscess, pus	9 yrs.; pyorrhea extensive	Spine, thoracic, and lumbar.
15	53466	Erythema, dietetic; pyorrhea	Alveolar abscess, pus	Several yrs.; pyorrhea; dietetic erythema, acute	
16	53465	Pulmonary tuberculosis; pyorrhea	Alveolar abscess, pus	Several yrs.; pyorrhea	
17	52512 (2)	Arthritis, chronic	Tonsil	6 mos.; chronic tonsillitis	Knees.
18	52631	Pyorrhea	Alveolar abscess, extraction	Pyorrhea, several years	
19	52796 (1)	Arthritis, chronic	Alveolar abscess, extraction	20 yrs.; extensive pyorrhea for 12 yrs.	All joints except spine and jaws.
20	52796 (2)	Arthritis, chronic	Alveolar abscess, extraction	20 yrs.; extensive pyorrhea for 12 yrs.	All joints except spine and jaws.
21	52779	Endocarditis, acute infective	Blood	Pyorrhea, several years	
22	53526	Endocarditis, chronic	Alveolar abscess, pus	Chronic endocarditis, duration not known	
23	53524	Endocarditis, chronic	Alveolar abscess, pus	Chronic endocarditis, duration not known; pyorrhea for several yrs.	
24	53668	Arthritis, acute, rheumatic	Alveolar abscess, pus	10 dys.; acute arthritis	Wrists, knees, ankles.
25	54669	Pyorrhea alveolaris	Alveolar abscess, pus	Pyorrhea, several yrs.	
26	51381	Myokymia	Pyorrhea, pus from gums	Pyorrhea, several yrs.; several yrs. recurrent attacks; myokymia for 6 mos.	Small joints and hands.
27	52513	Pyorrhea alveolaris	Alveolar abscess, pus from gums	Pyorrhea, several years	
28	55064	Tonsillitis, chronic	From abscess of tonsil after removal, pus	Chronic tonsillitis for several yrs.; acute abscess of tonsil; tonsillectomy and culture from abscess	

Antigen.	Laboratory No.	Diagnosis.	Source.	Duration.	Involvement.
29	52488	Arthritis and scrofula	Blood	6 mos.; *S. viridans* from blood	All joints ulcerating; probably tuberculosis; scrofula.
30	55240	Arthritis, chronic deforming	Pus from tooth after extraction	3 yrs.; pyorrhea for several yrs. 2 acute exacerbations; all joints involved within a year; *S. viridans* culture from pus from extracted tooth	All joints.
31 and 32	55241 55377	Arthritis, chronic; all joints	Pus from gums Tooth after extraction	9 yrs.; pyorrhea for yrs.; not considered serious by dentist; *S. viridans* from pus from gum and from extracted tooth	All joints.
33	55555	Arthritis, chronic; knees and ankles	Pus from gums	12 yrs.; knees, 12 yrs. and one ankle within last mo.; pyorrhea for years; *S. viridans* from pus from gum and from extracted tooth	Knees and ankle.
34	55854	Arthritis, chronic; knees and ankles	Tooth after extraction		Knees and ankle.
35	51189	Septicemia	Blood	3 wks.; supposed typhoid; *S. viridans* from blood, 3 cultures; cardiac signs normal; recovery under vaccine	

TABLE II.

Streptococci, Excepting Viridans, and Micrococci Used as Antigens.

Antigen.	Laboratory No.	Diagnosis.	Bacteria.	Source.
A			Gonococcus	Polyvalent antigen (Parke, Davis, & Co.).
B	48429	Arthritis deformans	*M. albus*	Cervix uteri.
C	48829	Arthritis deformans	*M. tetragenus*	Cervix uteri.
D	48831	Arthritis deformans	*M. tetragenus*	Cervix uteri.
E	50369	Adenitis, chronic infective	*M. aureus*	Tooth socket.
F	48923	Arthritis, chronic infective	*M. albus*	Sputum.
G	49416	Parkinson's disease	*M. aureus*	Prostate.
H	49765	Arthritis, acute	*S. hemolysans (hemolyticus)*	Tonsil.
I			*M. rheumaticus* (Beattie)	Stock.
K		Pneumonia, lobar	Pneumococcus	Stock culture, originally from sputum.
L		Septicemia	*S. hemolysans (hemolyticus)*	Blood.
M	53603	Arthritis deformans	*M. albus*	Urine.
N	46377	Endocarditis, chronic infective	*M. zymogenes*	Blood.
O	50251	Ulcerative rhinitis	*M. aureus*	Ulcer of nasal septum.

It was found that for *S. viridans* the best procedure is to dilute the bacterial emulsion to 1 c.c. (1,000,000,000). Of this emulsion from 0.8 to 0.1 c.c. and from 0.09 to 0.01 c.c. were tested for their inhibiting power (anticomplementary) against the standard sheep-rabbit hemolytic unit in 0.1 c.c. quantities with 0.1 c.c. of guinea pig complement corresponding to two units of hemolytic amboceptor and red cells. The complement and different amounts of antigen were incubated in a water bath at 37° C. for forty minutes, and the amboceptor and red cells added; the tubes were again incubated for forty minutes and placed on ice over night. The antigen to be used was selected from the highest limit of dilution of antigen which permitted complete hemolysis. It was found for *S. viridans* that the usable antigen, ten days after preparation, was from 0.03 to 0.01 c.c. (from 10,000,000 to 30,000,000) for the amounts of patients' serum and hemolytic unit used.

It was found that preservatives such as lysol and carbolic acid and alcohol could not be used since these substances interfered with the fixation of the antigen. Considerable difficulty, therefore, was encountered in preparing a permanent antigen. The antigens were kept cold, on ice, when not in use, and with precautions against contamination have often retained their activity for several months. They lose power with age. For a few days after preparation the antigen may be weaker, the usable quantity being as high as 0.09 c.c. (90,000,000), than after standing for a week or ten days, when the usable quantity will be found to be from 0.03 to 0.01 c.c. (30,000,000 to 10,000,000).

For the complement fixation tests 0.03, 0.02, and 0.01 c.c. of patients' serum were used with the amounts of antigen and hemolytic unit previously noted, and

controls were prepared with the same amounts of patients' serum and hemolytic unit and complement, without antigen.

The amount of complement-fixing body (*fixateur*) in patients' serum was found to be small, for often only a partial inhibition of hemolysis occurred with 0.01 c.c. of serum (0.1 c.c. of 1:10 dilution), while complete inhibition occurred with 0.02, 0.03, 0.04, and 0.05 c.c. of a positive serum which was weak in anti-complementary power.

The necessity for the employment of so many streptococcus antigens is due to the facts that a group reaction for several strains could not be obtained; without preservation it is difficult to prevent contamination, and the retention of fixability is variable. As a rule antigens freshly prepared and two or three weeks old give good fixation, within narrow limits.

It has not always been possible to carry through a known positive serum, but several sera have been tested at a time and a known negative serum used as a control, the negative or positive nature of the control sera having been tested in comparison with rabbit sera, recorded in the report on animal experiments (9).

COMPLEMENT FIXATION TESTS IN ARTHRITIS. RESULTS POSITIVE FOR STREPTOCOCCUS VIRIDANS. SEVENTEEN CASES.

In table III are recorded the results of positive complement fixation tests for *S. viridans* with sera from patients afflicted with chronic infective deforming arthritis. The qualification "chronic infective deforming" is applied because the positive reactions indicate the infective nature of the disease. Cases 1, 8, 9, 10, and 16 began as mildly febrile infections in one or more joints, rapidly extended within a few weeks to most of the large and small joints, and ultimately produced the joint deformities—and the joint lesions shown by radiographs in cases 1 and 10,—found in classical cases of arthritis deformans.

Cases 8 and 9, with hepatomegaly and splenomegaly, in females of nine years, were typical of Still's Disease.

Cases 7, 10, 13, and 14 only were males; the remaining thirteen were females. Ages ranged from 30 (case 15) to 69 years (case 16). Duration, at first visit, was from 9 months (case 1) to 30 years (case 5). In fifteen cases, all except cases 7 and 13, foci of infection were found. Cultures of *S. viridans* were isolated from eleven cases, in ten from the teeth, and in one from the sputum. In one case (No. 3), *Micrococcus tetragenus* was isolated from uterine curettings, and in five cases no cultures were taken. In four cases with no cultures there was a history of pyorrhea alveolaris in three and a history of acute rheumatism in one.

TABLE III.

Sera Reacting to Streptococcus viridans.

Cases of arthritis deformans and Still's disease.	Age in yrs.	Date.	Wassermann reaction.	Gonococcus infection.	Cases of infective deforming arthritis: bacterial antigens.																														Culture from foci.
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	A	B	C	D	E	F	G	H	I	K	N	
1. Mrs. C.	38	vi–11	0	0	o	+																													*S. viridans* + from abscess of jaw.
						Autogenous																														
		v–13	0	0	o	o			o			o			o	o		+	o					o	o	o						o	o	
2. Mrs. L.	41	i–3–12	0	0		+	+ o	o																											*S. viridans* + from tooth socket and sacculi.
						Autogenous																														
		i–15–12	0	0	o	+	+																		o										
3. Mrs. B.	43	ii–12	0	+		+ o	+	o			+	o	+	o											+	o			+	o					*M. tetragenus* from cervix and curettings.
4. Mrs. S.	40	iv–12	0 +	0	o	o	o		+	o	o														o	o		o	o	o					*S. viridans* + from pus from gums.
5. Miss L.	60	v–12	0	0	o+	+			o	o	o	o													o	o		o	o	o	+ o				*S. viridans* from tooth extracted.
6. Mrs. W.	40	x–12	0	0	o	o	+		o		+	o													o	o		o	o	o	+	o	o		*S. viridans* + from tooth pulp, socket, sacculi from gum.
		iv–13	0	0	o	o			o			o		+		o																			
														Autogenous																						
7. F. S.	41	iii–12	0	+	+																			o											None.
8. O'D.	9	ii–12	0	0		+			+					+										o								o			*S. viridans* from pus from gums. Still's disease.
9. S.	9		0	0									+																						*S. viridans* from pus from gums. Still's disease.
														Autogenous																						
10.[1] P.	49	ii–12	0	+		o	+ o	+ o	+															+	o			+ o	o					*S. viridans, B. influenzæ, M. aureus* in sputum.
								Autogenous																												
11. R.	48	vi–13	0	0			o	o	o	+	o	o	o	o	o									o											Alveolar abscess, cured; no cultures.
12. M.	50	iii–13	0	0	o	+						o												o			o	o	+	o		o			Pyorrhea alveolaris, cured; no cultures.
13. S.	?	v–13	0	0	o	o			o			o			o	o								o	o		o					+	o		Acute rheumatism years ago; no cultures.
14. C.	46	x–13	0	0											o				+				+	o							+		o	Pyorrhea alveolaris, cured; no cultures.
15. G.	30	x–11	0	0																				o											Pyorrhea alveolaris, cultures from teeth and *M. albus* from cervix uteri.
		xi–11	0	0																				o										
		xi–13	0	0											o				+				+	o								o		o

Cases of arthritis deformans and Still's disease.	Age in yrs.	Date.	Wassermann reaction.	Gonococcus infection.	Cases of infective deforming arthritis: bacterial antigens.													Culture from foci.
					1	18	20	25	28	29	30	31	32	33	34	36	I	N	
16. Mrs. B.	69	xi–13	0	0	0	...	...	+	..	+	..	.....	.....	.....	.....	.....	0	0	Pyorrhea alveolaris, extensive; *S. viridans* from pus from gums and from tooth sockets.
17. Mrs. McM.	60	ii–14	0	0	0	0	0	...	+	0	0	0	+	+	0	+	0	0	Pyorrhea alveolaris, extensive; *S. viridans* from pus from gums and from tooth sockets.

[1] Case 10 gave also a positive reaction for gonococcus infection (table IV, case 20), and improved under gonococcus vaccine. The positive fixation test to the autogenous antigen (No. 5) of *S. viridans* from the sputum was probably due to the chronic infection in the bronchi.

In two cases not recorded since the blood was not tested for complement fixation, *S. viridans* was isolated from the prostate.

In five cases (1, 2, 6, 9, and 10) tests against autogenous antigens of *S. viridans* were positive. Two cases (13 and 14) gave positive reactions against *M. rheumaticus.* Cases 3, 7, and 10 gave positive tests for gonococcus infection and thus caused confusion in respect to a choice of vaccine for specific therapy. Cases 3 and 7 did not improve under injections of gonococcus vaccine and improved under *S. viridans* vaccine; case 10 improved under gonococcus vaccine, and the positive reaction to autogenous *S. viridans* might be explained by the infection in the bronchi, since *S. viridans* was isolated from the sputum. Treatment of cases 7 and 10 resulted in what should be termed a cure, with deformity but with unusual restoration of function. Cases 3, 5, 6, and 10 gave positive tests for *M. aureus,* and case 12 for *M. albus;* in case 10 only, with a positive culture of *M. aureus* from the sputum, could a probable focus of infection for *M. aureus* be found. What relation these micrococci might have had to the arthritis was not determined.

ABSTRACTS OF CASES OF ARTHRITIS DEFORMANS GIVING POSITIVE FIXATION TESTS FOR STREPTOCOCCUS VIRIDANS.

Case 1.—Mrs. C., aged 38 years. Duration, at first visit, June, 1911, 9 months. Began in knees after exposure to wet and cold (rheumatic throat) in 1908; abscess of left lower maxilla under last molar tooth. Feb., 1912. Clinical course of infective disease with pyrexia for seven months; all joints involved including cervical spine and both temperomaxillary joints; periarthritis; tenosynovitis; neuritis; phlebitis of left saphenous and popliteal. Oct., 1913, condition typical of arthritis deformans.

Case 2.—Mrs. L., aged 41 years. Duration, at first visit, Jan. 1, 1912, 12 years. Began in small joints of hands one month after parturition with pyrexia; persistent endometritis; all joints including cervical spine; periarthritis; tenosynovitis; neuritis. *S. viridans* from tooth extracted in 1912.

Case 3.—Mrs. B., aged 43 years. Duration, at first visit, Feb., 1912, 4 years. Began insidiously in knees, ankles, and wrists; persistent endometritis; all joints including cervical spine and left temperomaxillary joint; periarthritis; tenosynovitis; neuritis; phlebitis of left saphenous vein; pleuritis.

Case 4.—Mrs. S., aged 40 years. Duration, at first visit, Apr., 1912, 4 years. Began insidiously in knees and wrists; pyorrhea alveolaris for years; all joints involved, except spine and jaws.

Case 5.—Miss L., aged 60 years. Duration, at first visit, May, 1912, 30 years. Began insidiously in fingers, elbows, and knees; pyorrhea alveolaris for years,

whether or not preceding arthritis is not known; all joints including cervical spine and temperomaxillary joints; periarthritis; tenosynovitis; neuritis; extensive crippling and marked deformity. Thrombophlebitis and gangrene of one leg and toes. Death, Feb., 1914.

Case 6.—Mrs. W., aged 40 years. Duration, at first visit, Oct., 1912, 1 year. Began in left temperomaxillary joint; radiograph showed apical abscess over left upper molar; mild polyarthritis.

Case 7.—F. S., male, aged 41(?) years. Duration, at first visit, Mar., 1912, 1½ years. Began in ankle and wrists; one elbow, small bones of right hand, and small joints of feet involved; chronic prostatitis; gonococcus vaccine for three months before first visit; no improvement; *S. viridans* vaccine; marked improvement and cessation.

Case 8.—O'D., female, aged 9 years. Duration, at first visit, Feb., 1913, 3 years. Still's disease; all joints involved, except spine; deformity typical of arthritis deformans.

Case 9.—S., female, aged 9 years. Duration, at first visit, Nov., 1912, 2 years. Still's disease; all joints involved, except spine; deformity typical of arthritis deformans.

Case 10.—P., male, aged 49 years. Duration, at first visit, Feb., 1912, 2 years. Previously tonsils and teeth inspected and teeth treated; tonsils not treated. Began in wrists, ankles, and knees; all joints involved except hips and temperomaxillary joints; complement fixation tests positive to gonococcus and *S. viridans;* deformity more typical of gonococcus arthritis than of arthritis deformans; recovery under treatment with gonococcus vaccine.

Case 11.—Mrs. R., aged 48 years. Duration, at first visit, June, 1913, 12 years. Began in toes, extended to all joints of left foot and then right foot; could not walk for a year; rheumatism 18½ years before first visit, during pregnancy and puerperium; all joints involved except the spine; left internal ear painful,—diagnosed "rheumatic ear"; radiograph showed apical abscess of right upper molar, which was treated and pronounced cured one month before first visit; deformity moderate.

Case 12.—Miss M., aged 50 years. Duration, at first visit, Feb., 1913, 5 years. Began in left knee; shortly followed to right knee and all joints, excepting left jaw and hips; progress slow with exacerbations of pain, redness, increased swelling, and local heat in joints; deformity moderate.

Case 13.—S., male, age not known. Duration, at first visit, May, 1913, several years, following acute rheumatic arthritis years before; all joints involved; deformity typical of arthritis deformans.

Case 14.—T. C. C., male, aged 46 years. Duration, at first visit, Oct., 1913, 8 years. Began in knees; all joints except right shoulder involved; deformity and contractures marked; perisynovitis; neuritis and extreme emaciation.

Case 15.—Mrs. R. G., aged 30 years. Duration, at first visit, Nov., 1911, 8 years. Began in knees and wrists; all joints, except spine and hips, involved; pyorrhea alveolaris and endometritis for years; arthritis slowly progressive with exacerbations.

Case 16.—Mrs. B., aged 69 years. Duration, at first visit, Nov. 29, 1913, 6 years. Acute rheumatism at 6 years of age; chronic arthritis began in 1908;

an acute attack with low fever for a few days and inflammation of left thumb, followed by small joints of both hands, wrists, shoulders, hips, knees, maxillary joints, cervical spine, and ankles; course progressive with exacerbations; neuritis in left arm two years ago; teeth considered excellent until two years ago; considered good at present, although pyorrhea is extensive, involving six or more teeth; indigestion and foul stools for years. Died of Bright's disease and uremia, Mar., 1914.

Case 17.—Mrs. McM., aged 60 years. Duration, at first visit, Feb. 12, 1914, several years. Began in ankles and knees; involved subsequently small joints of hands and feet, spine, shoulders, elbows; pyorrhea alveolaris extensive for several years.

In the same case one may find hypertrophic, atrophic, periarthritic, and often tenosynovitic lesions; and multiplicity of lesions is noticeable in the acute and chronic infective cases which end ultimately in the progressive deforming arthritis without evidences of infection. In two cases only, not recorded in table III, since the progress of the disease and the deformity were not of arthritis deformans, have the symptoms and signs been those of an atrophic arthritis confined to the joint surfaces, with creaking and crepitation on motion, little tenderness, no pain, no local temperature, and with ultimate erosion of the small joints; one was a female, aged 44 years, and one a male, aged 40 years, and in both the progress was slow, over a period of three or four years with no annoyance other than that from the creaking and grating and the partial loss of control of the small joints.

RESULTS POSITIVE FOR GONOCOCCUS.

Table IV gives the results with eight cases reacting to gonococcus antigen. It may be assumed that the tests in cases 18 and 19 were incomplete since no *S. viridans* antigens were used. Four cases (18, 19, 20, and 21) exhibited deformity characteristic of arthritis deformans. Two of these four cases (Nos. 2 and 21) were classed as infective deforming arthritis and are included under the cases in table III, since both reacted also to *S. viridans*.

Table V records three cases of multiple arthritis, giving positive Wassermann reactions and recovering under antisyphilitic treatment. The positive gonococcus reaction in case 26 (table IV, case 24) indicated no causative relation to the disease.

TABLE IV.

Sera Reacting to Gonococcus Antigen.

Cases of arthritis deformans.	Wassermann reaction.	Gonococcus.	Bacterial antigens.															Culture from foci.
			1	2	3	4	5	6	7	8	9	10	11	A	B	E	F	
18. J. P.	0	+												+				None.
19. K.	0	+												+				None.
20. P.[2] (10)	0	+	0	0	+ 0	+ 0	(+ Autogenous)							+	0	+ 0	0	*S. viridans* from sputum. Staphylococcus and *B. influenzæ* from sputum.
21. Mrs. B.[3] (3)	0	+		+ 0	+	0			+	0	+	0		+	0	+	0	*M. tetragenus* from cervix uteri.
Infective deforming arthritis.																		
22. H.	0	+			0									+		0		*M. albus* from bladder.
23. C.	0	+		0					0	0				+				None.
24. D.[4] (26)	+	+	Luetic arthritis											+	0			None.
25. P.	0	+	0		0	0		0	0	0	0	0	0	+				None.

TABLE V.

Sera Reacting for Lues.

Polyarthritis, periostitis, luetic.	Wassermann reaction.	Gonococcus.	Bacterial antigens.												Culture from foci.
			1	2	3	4	5	6	7	8	D	E	F	H	
26. D.[5]	+	+							0	0					None.
24.															None.
27. E.	+	0	0	0	0		0		0	0		0			None.
28. W.	+	0	0	0	0		0	0	0	0	0	0	0	0	None.

COMPLEMENT FIXATION TESTS IN ARTHRITIS. RESULTS NEGATIVE.

Table VI records the tests in eighteen cases of arthritis which should be classed as arthritis deformans. In eight cases (Nos. 29,

[2] Case 20 gave a positive reaction for *S. viridans* and is recorded in table III also (case 3).

[3] Case 21 gave a positive reaction for *S. viridans* and is recorded in table III also (case 10).

[4] Case 24 gave a positive Wassermann reaction and is recorded in table V also; recovered under antisyphilitic therapy.

[5] D. gave a positive reaction for gonococcus infection and is recorded also in table IV. A fourth case (A. L., female) giving a positive Wassermann reaction is not recorded since the arthritis was monarticular (Charcot's joint).

30, 31, 32, 33, 34, 35, and 36) tests against *S. viridans* were not made, and it might be assumed that some of these would give positive results.

Summary.—Of 43 cases[6] of deforming arthritis, 25 (58 per cent.) gave positive evidence by complement fixation and Wassermann tests of their infective nature; 19[7] cases (44 per cent.) of arthritis de-

TABLE VI.

Arthritis Deformans. Sera Not Reacting to Complement Fixation Tests.

Case No.	Cases of arthritis deformans.	Wassermann reaction.	Gonococcus infection.	Bacterial antigens. 1	2	3	4	5	6	7	8	9	10	11	12	B	D	E	F	G	H	I	K	O
29	H. McN.	0	0																					
30	B. McG.	0	0(2)																					
31	M. B.	0	0(2)																					
32	A. H.	0	0																					
33	J. T.	0	0																					
34	A.K. ix–10	0	0																					
	vi–11	0	0																					
35	E. G.	0	0(2)		o							*M. tetragenus* from cervix uteri.												
36	R.	0	0																					
37	H.	0	0	o	o		o			o	o		o											
38	McM.	0	0	o	o			o			o			o	o							o		
39	Mrs. M.[8]	0	0	o	o	o		o	o	o	o					o	o	o	o	o				o[9]
40	P. ix–12	0	0	o	o	o		o		o	o		o		o		o	o	o	o	o			
	x–12	0	0	o	o	o		o					o				o	o	o					

Case No.	Cases of arthritis deformans.	Wassermann reaction.	Gonococcus infection.	Bacterial antigens. 1	2	5	8	11	12	14	15	16	17	18	19	20	21	22	A	B	I	K
41	S.	0	0	o	o	o	o	o	o	o[9]	o										o	o
42	B.	0	0	o	o	o	o	o	o			o								o	o	
43	P.	0	0	o	o	o		o	o	o	o	o	o	o								o
44	H.	0	0	o	o	o		o	o	o	o	o	o	o								o
45	D.	0	0									o			o	o	o	o				
46	S.	0	0						o			o			o	o	o[9]	o			o	

formans (including 2 cases of Still's disease) gave positive tests, and of these 19 cases, 17 (39 per cent.) reacted positively to *S. viridans* antigens, and 4 (9.3 per cent.) positively to a polyvalent gonococcus antigen.

[6] Total in tables, 46 cases; as 3 cases are counted twice, the total number of cases is 43.

[7] Total in tables, 21 cases; as 2 cases are counted twice, the total number of cases is 19.

[8] Negative to autogenous antigen (*S. aureus*) from ulcer of nasal septum.

[9] Autogenous.

Three cases of multiple arthritis and periostitis of the small joints of the hands and the distal ends of the long bones of the forearms and legs reacted to the Wassermann test and recovered under antisyphilitic therapy.

Summary.—Of 37 cases of arthritis deformans, recorded in table IV, exclusive of case 10, and three cases of luetic arthritis and four cases of infective deforming arthritis due to gonococcus, 19 (51 per cent.) gave positive reactions for complement fixation; of these 19 cases, 16 (43 per cent.) with *S. viridans,* and 3 (8.1 per cent.) with gonococcus antigens.

Blood Cultures.—Cultures of blood withdrawn from the median vein were negative in every case, but little importance can be attached to the negative results, since in two cases only was more than one culture taken.

SUMMARY OF CASES.

Positive.	Complement fixation.	
Arthritis deformans	Cultures from foci.	Autogenous antigen.
S. viridans[10]	15 cases	Positive in 5 cases.
16 cases	2 cases	
Gonococcus[11]	(Not gonococcus)	
3 cases		
Chronic infective deforming arthritis		
Gonococcus[12]		
4 cases		
Wassermann		
3 cases		
Total 26		
Negative.		
Arthritis deformans		
18 cases		
Total cases 44		

COMPLEMENT FIXATION ON CONTROL CASES.

NOT ARTHRITIS.

Table VII records complement fixation tests for strains of *S. viridans* and for a polyvalent gonococcus antigen and Wassermann

[10] Case 70 proved to be a gonococcus infection and is taken from the 17 cases in table III and placed in table IV (case 22).

[11] Case 21 proved to be a *S. viridans* infection and is taken from the five cases in table IV and placed in table III (case 3).

[12] Case 24 proved to be a case of luetic arthritis and is taken from the four cases in table IV and placed in table V (case 26).

TABLE VII.

Controls.

Case No.	Diagnosis.	Wassermann reaction.	Gonococcus.	Bacterial antigens.																																			Remarks.
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	21	23	25	28	29	30	31	A	B	C	E	F	G	I	K	N	
47	Acute rheumatic arthritis	0	0	o	o						o		o																				o		+	o		
48	Acute rheumatic arthritis	0	0	o																		o			o	o	o	o	o							o			
49	Acute rheumatic arthritis	0	0	o																		o			o	o	o	o	o							o			
50	Acute rheumatic arthritis	0	0	o	o							o				o					o	o			o					o	o	o							*S. viridans* from pus and from gums; autogenous antigen 24, anticomplementary.
51	Acute infective endocarditis, malignant	0	0	o	o		o																							o		o	o						*S. viridans* from blood.
52	Acute infective endocarditis	0	0	o	o		o																							o		o	o						*S. viridans* from blood.
53	Chronic infective endocarditis	0	0	o	o		o																																*S. viridans* from blood.
54	Septicemia	0	+	o	o																																		*S. hemolyticus* from blood.
55	Chronic endocarditis, aortic	+	0	o	o								o	o		o																			o	o		
56	Chronic endocarditis, v–12–13, mitral	0	0	+	o							o				o					o	o	o	o						o	o	o							*S. viridans* from pus from gums; autogenous antigen 22, anticomplementary.
	v–19–13, mitral	0	0	+	o							o				o					o	o	o	o						o	o	o						
57	Chronic endocarditis, (with nephritis) v–12–13	0	0	o	o							o				o					o	o	o	o						o	o	o							*S. viridans* from pus from gums; autogenous antigen 23, negative.
																								Autogenous															
	v–19–13	0	0	o	o							o				o					o	o	o	o						o	o	o						
58	Chronic endocarditis	0	0	o	o			o			o			o	o																				o			

Case No.	Diagnosis.	Wassermann reaction.	Gonococcus.	Bacterial antigens.																																			Remarks.
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	21	23	25	28	29	30	31	A	B	C	E	F	G	I	K	N	
59	Acute tonsillitis	o	+	o	o	o	.	.	.	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*S. viridans* from tonsil; antigen 7; prostatitis.
60	Chronic infective adenitis (ii-18-12), simulating Hodgkin's disease (vi-20-13)	o	o	−	o	+	o	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	.	o	o	.	.	.	.	.	*S. viridans* from pus from gums and from extracted teeth.
		o	o	+	o	+	−	+	o	o	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	o	.	.	.	.	.	
61	Pyorrhea alveolaris, influenza	o	o	o	o	.	.	o	.	.	o	.	.	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	.	.	.	
62	Pyorrhea alveolaris, lobar pneumonia	o	o	o	o	.	.	o	.	.	o	.	.	o	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	o	o	.	.	.	o	o	.	.	
63	Pyorrhea alveolaris, pulmonary tuberculosis	o	o	o	o	.	.	o	.	.	o	.	.	o	o	.	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	o	.	.	*S. viridans* from pus from gums.
64	Pyorrhea alveolaris, pulmonary tuberculosis.	o	o	o	o	.	.	o	.	.	o	.	.	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	+	.	.	
65	Pyorrhea alveolaris, erythema	o	o	o	o	.	.	o	.	.	o	.	.	o	o	.	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	o	.	.	*S. viridans* from pus from gums; autogenous antigen 14, negative.
																		Autogenous																				
66	Anemia, secondary	o	+	o	o	o	.	.	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	o	o	.	.	.	.	
67	Myeloma, Bence-Jones proteinuria	o	o	o	o	.	.	o	.	.	.	.	.	o	o	.	o	o	o	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	.	.	Osteoporosis of spine; Bence-Jones proteinuria.
68	Sciatica	o	o	o	o	.	.	.	.	.	o	.	.	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	o	.	o	o	.	.	
69	Lumbago	o	o	o	o	.	.	o	o	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	.	.	.	.	
70	Lumbago	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	.	.	o	.	o	.	.	.	.	.	.	.	.	o	.	.	o	Lumbago (spondylitis?), neurasthenia.
71	Paralysis agitans	o	+	o	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	o	o	o	o	.	.	.	.	.	.	.	.	.	.	*M. albus* from prostate.
72	Paralysis agitans	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	o	.	.	o	.	o	.	.	.	.	.	.	.	.	o	.	.	o	
73	Myasthenia gravis	o	o	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

tests in a miscellaneous group of cases not afflicted with arthritis. One (case 47) of four cases (Nos. 47, 48, 49, and 50) gave a positive reaction against *M. rheumaticus.* Two cases (Nos. 50 and 51) of acute infective endocarditis gave negative tests at the time that the *S. viridans* was isolated from the blood. Case 54, of septicemia, gave a positive fixation test for gonococcus, and *Streptococcus hemolyticus,* the causative agent, was isolated from the blood. Three other cases (Nos. 59, 66, and 71), one of acute tonsillitis and chronic prostatitis, one of secondary anemia, and one of paralysis agitans, in males, gave positive reactions against gonococcus, and as with case 54 the positive blood reactions bore no relation to the affliction complained of and should be considered as positive evidence of clinically latent gonococcus infection which one might suppose to be relatively common on account of the high incidence of gonococcus infection, particularly in the male. In this case of acute tonsillitis (No. 59) *S. viridans* was isolated from the surface of the tonsil, and the complement fixation test for *S. viridans* was negative.

One case (No. 55) of chronic endocarditis involving the aortic valve gave a positive Wassermann reaction, and one (No. 56) involving the mitral valve gave twice a positive reaction to *S. viridans* (antigen 1).

In one case of multiple adenitis, simulating Hodgkin's disease, the blood reacted positively to three strains (antigens 1, 3, and 5) of *S. viridans,* but not to autogenous antigen (No. 4) of *S. viridans* from pus from the gums and from the sacculi on extracted teeth.

One case (No. 64) of pulmonary tuberculosis with secondary infection, and with extensive pyorrhea alveolaris reacted to the pneumococcus, possibly in response to the secondary infection in the lung, which can be surmised only since there is no record of a culture from the sputum.

Cases 61, 62, 63, 64, and 65, of extensive pyorrhea alveolaris and suffering from and under observation for diseases undoubtedly not due to a type of streptococcus, were negative to tests for *S. viridans.*

On the whole, it should be noted that the 26 control cases were consistently negative to complement fixation tests for *S. viridans* and that the three cases (Nos. 47, 56, and 60) which reacted to

M. rheumaticus and *S. viridans,* two organisms closely related if not strains of one organism as will be shown in another paper by Dr. Thro, were probably infected with strains belonging to the *S. viridans* group.

CONCLUSIONS.

1. From a comparison of the results with arthritis deformans recorded in tables III, IV, and VI, and with the control cases recorded in table VII, one is justified in concluding that *Streptococcus viridans* is an infectious agent and excites the production of a complement-fixing substance (*fixateur*) in the organism in cases of arthritis deformans, and, therefore, *Streptococcus viridans* is the probable causative agent of the disease in many cases of arthritis deformans. Probably 40 per cent. and more of cases of arthritis deformans should be considered as chronic infective deforming arthritis.

2. Rarely the clinical manifestations of arthritis deformans may be due to gonococcus infection.

3. The serum from one case may react positively to two different organisms, most frequently to *Streptococcus viridans* and to gonococcus, when the reaction to the former should be considered the indicator of the causative agent, since with the latter infection is often latent in the genito-urinary tract. Incidence of gonococcus infection is high and the clinical manifestations of arthritis deformans are rarely produced by gonococcus infection.

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CONCERNING A POLYVALENT ANTIGEN FOR THE COMPLEMENT FIXATION TEST FOR STREPTOCOCCUS VIRIDANS INFECTION.*

By T. W. HASTINGS, M.D.

(From the Department of Clinical Pathology of Cornell University Medical College, and The Second Medical Division of Bellevue Hospital, New York.)

The following experiments were conducted to determine the time of appearance of complement fixation substances in rabbits. In making control tests with normal rabbits before streptococcus vaccine was injected, it was found that rabbits may have in small amount a natural *fixateur* to *Streptococcus viridans,* since some tests were positive. Normal rabbits did not react to staphylococci. Complement fixation became positive in eleven days. The disturbance due to the anticomplementary action of some rabbit serum is well known.

Mar. 8, 1913, 11 A. M. Seven mice were inoculated as shown in table I. Antigen 2 was prepared from a few days' growth of *S. viridans,* isolated from an abscess of the left lower jaw in a case of acute infective deforming arthritis, later typical arthritis deformans. The growth was suspended in 0.8 per cent. saline solution and killed by heating to 60° C. for thirty minutes and standardized by Wright's method and diluted so that 1 c.c. contained 100,000,000 streptococci. Antigen 11 was isolated from an alveolar abscess in a case of arthritis deformans and was prepared as antigen 2. Antigens 2 and 11 had been used for the vaccinations of cases of arthritis deformans and had given positive complement fixation tests with blood from patients suffering with arthritis deformans.

TABLE I.

Mouse No. Mar. 8, 1913.	Antigens killed by heating.				Result.
	S. viridans 2.	*S. viridans* 11.	*M. rheumaticus* (Beattie).	Pneumococcus.	
275	10,000,000				No effect.
259	50,000,000				No effect.
251		10,000,000			No effect.
276		50,000,000			No effect.
236			50,000,000		No effect.
260				10,000,000	No effect.
252				50,000,000	No effect.

* Received for publication, April 20, 1914.

The *M. rheumaticus* antigen was a strain obtained from the New York Board of Health Laboratories and originally came from the laboratory of Dr. J. M. Beattie, of Edinburgh. The pneumococcus antigen was prepared from a moderately virulent strain obtained from the blood of a case of lobar pneumonia. The *M. rheumaticus* and pneumococcus antigens were prepared and standardized and diluted as antigens 2 and 11.

Mar. 25, 1913. The same seven mice were inoculated as shown in table II. Antigen 10 was prepared from a culture from the tonsil of a case of infective arthritis. All the antigens were ten times the strength of those inoculated on Mar. 8, and five were killed by heating to 60° C. for thirty minutes. Antigen 13 and pneumococcus were isolated from the blood, grown on North's medium and not killed. The pneumococcus antigen was from a twenty-four hour growth, and was the same as the pneumococcus antigen killed by heating to 60° C.

TABLE II.

Mouse No. Mar. 25, 1913.	Antigens killed by heating.					Antigens not killed.		Result.
	S. viridans 2.	*S. viridans* 10.	*S. viridans* 11.	*M. rheumaticus.*	Pneumococcus 10.	*S. viridans* 13.	Pneumococcus 10.	
275	500,000,000							No effect.
259		500,000,000						No effect.
251			500,000,000					No effect.
276				500,000,000				No effect.
236					500,000,000			No effect.
260						500,000,000		No effect.
252							500,000,000	Death after 72 hrs.

The mouse inoculated with the living twenty-four hour culture of pneumococcus died after seventy-two hours. The other inoculations in doses of 500,000,000 were without effect, even the inoculation of 500,000,000 living *S. viridans*.

On Mar. 28, three days after the injection of 500,000,000, two of the mice, Nos. 259 and 251, were inoculated with 0.75 c.c. of living polyvalent antigen prepared from fourteen strains of *S. viridans* of five days' growth. The dosage in millions by Wright's method was not determined, but was several times stronger than that of Mar. 25, since the cultures were grown on 2 per cent. glycerin agar tubes from 2 to 3 cm. in diameter, and the surfaces of these fourteen large tubes were washed with 20 c.c. of saline solution. For the 500,000,000 dilution the surface of a small agar tube was washed with 2 c.c. of saline solution.

It is safe, therefore, to assume that the stronger antigen was ten times 500,000,000, or 5,000,000,000. The possibility of immunity in the mice from previous injections, on March 8 of 50,000,000, and on March 25 of 500,000,000, was wanting, since the 500,000,000 dose which might have caused such immunity was given three days before the dose of 5,000,000,000. From the dose of 50,000,000 on March 8, after an interval of twenty days one would expect

anaphylaxis rather than immunity. Subsequently these mice were injected again with 5,000,000,000 living *S. viridans* without any apparent effect.

The virulence of living *S. viridans* having proved impotent for mice, rabbits were inoculated intravenously with large doses of living antigen, prepared from fourteen strains grown in large tubes of 2 per cent. glycerin agar for four days. On May 28 four rabbits were inoculated, as shown in table III, three with one third of the antigen, and a fourth with the saline solution suspension of the growth of *M. albus* from one agar tube.

TABLE III.

Rabbit No. May 28, 1913.	Polyvalent antigen not killed, 14 strains, *S. viridans*.	*M. albus* antigen not killed, 1 agar culture in 1 c.c.
521 intravenous	5.0 c.c.	
482 intravenous	5.0 c.c.	
244 intravenous	5.0 c.c.	
498 intravenous		1.0 c.c.
Repeated June 5	7.0 c.c.	1.0 c.c.
Repeated June 11	8.0 c.c.	2.5 c.c.
Repeated June 19	8.0 c.c.	3.0 c.c.

On June 3, six days after the first inoculation, blood was examined and the rabbit serum tested against eleven of the fourteen strains for immunization, for complement fixation substance, with the results shown in table IV.

For the complement fixation tests the serum was diluted 1:10, and 0.3, 0.2, and 0.1 c.c. were used in three tubes, indicated by the three rows of + or 0 signs.

The antigens were killed by heating to 60° C. for half an hour, standardized in the usual manner in the forenoon of the day on which they were used, and the strength in millions was determined by Wright's method. It was found that as a rule an antigen of small strength test (1 c.c. = 1,000,000,000) should be diluted 1:10, and from 0.3 to 0.1 c.c. used against from 0.3 to 0.1 c.c. of serum.

From table IV it is seen that six days after a single inoculation of large doses of living *S. viridans* polyvalent antigen, the complement fixation substance is present in rabbit serum in small amount, and that the reactions are variable and incomplete against each of the strains used for immunization.

On June 5, 11, and 19, the same four rabbits were inoculated in the same manner; three with the living antigen prepared from fourteen strains of *S. viridans* and one with *M. albus* antigen.

TABLE IV.

Rabbit No.	Wassermann reaction.	Gonococcus.	*S. viridans* antigens.										
			1	2	3	4	5	6	7	8	9	10	11
521	+	+	Inhibition in control	Inhibition in control	o	+ oo + + o + + +	o	o	+ oo + oo + oo	+ oo + + o + + +	ooo + oo + + o	+ oo + oo + + +	+ + o + + + + + +
482	+	+	Inhibition in control	Inhibition in control	+ + o + + + + + +	+ + o + + + + + +	+ + o + + + + + +	ooo + oo + + +	o	+ + o + + + + + +	ooo + + o + + +	+ + o + + + + + +	+
244	+	+	Inhibition in control	Inhibition in control	+ oo + oo + + o	+ oo + + o + + +	+ + o + + o + + o	ooo + oo + oo	+ oo + + + + + +	+ oo + oo + + +	+ + o + + + + + +	ooo ooo + + o	+ + o + + + + + +
498 *M. albus*	+	+	+	+	+	+	+	+	+ + o + + + + + +	+	+	+	+

+ indicates slight hemolysis; + +, marked hemolysis; + + +, complete hemolysis; o indicates inhibition. A single large + or o indicates complete hemolysis or inhibition in three tubes.

On June 18, seven days after the third inoculation, the rabbit serum was tested with the results shown in table V.

TABLE V.

Rabbit No.	Wassermann reaction.	Gonococcus.	Compound antigen.		Antigen 5.
			1	2	
521	+	+	+	+	+
482	+	+	+	ooo + oo + + o	+ oo + + + + + +
244	+	+	+	+	+
498	+	+	+	+	ooo + oo + + +

The rabbit serum was found to inhibit, as shown in table VI, and this anti-complementary action was neutralized by using two units (0.2 c.c.) of complement.

TABLE VI.

Rabbit serum 1:10.	Fresh guinea pig complement, 1:10.			
	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
521	ooo ooo ooo	+	+	+
482	ooo ooo ooo	+	+	+
244	ooo ooo ooo	+	+	+
498	ooo ooo ooo	+	+	+

The hemolytic unit used was the rabbit-sheep system so standardized that 0.1 of a cubic centimeter of fresh guinea pig serum gave complete hemolysis in twenty minutes, and 0.05 of a cubic centimeter complete hemolysis in one hour in the water bath at 37° C.

At this time compound antigens prepared from the fourteen strains were used instead of the eleven single antigens used on June 3. An attempt was made to pool the antigens and thus employ a

TABLE VII.

Antigen No.					
1	*S. viridans*	14 strains	In 0.8% saline	Heated to 60° C. for 30 min.	
2	*S. viridans*	14 strains	In 0.8% saline	Heated to 60° C. for 30 min.	Chilled, digested, filtered.
3	*S. viridans*	14 strains	In distilled water	Heated to 60° C. for 30 min.	
4	*S. viridans*	14 strains	In distilled water	Heated to 60° C. for 30 min.	Chilled, digested, filtered.
5	*M. albus*	1 strain	In 0.8% saline	Heated to 60° C. for 30 min.	
6	*M. albus*	1 strain	In 0.8% saline	Heated to 60° C. for 30 min.	Chilled, digested, filtered.
7	*M. albus*	1 strain	In distilled water	Heated to 60° C. for 30 min.	
8	*M. albus*	1 strain	In distilled water	Heated to 60° C. for 30 min.	Chilled, digested, filtered.

single antigen for the detection of infection by any one of several strains, as was done by Schwartz and McNeil for the gonococcus.

Four antigens were prepared from a mixture of fourteen strains of *S. viridans:* two of them in saline solution were heated to 60° C. for thirty minutes and one of these was chilled on ice over night, digested in the thermostat at 37° C. for twenty-four hours, and filtered through a Berkefeld filter; two of them in distilled water were heated to 60° C. for thirty minutes, and one of these was chilled, digested, and filtered. Similarly four antigens were prepared from the *M. albus* strain and standardized (tables VII and VIII).

TABLE VIII.

Dilution antigen No. June 19, 1923.	Undiluted.						Diluted 1:10.						Diluted 1:100.						Amount used for tests.
	0.9 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.9 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.9 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	
1					+ + o	o	o	+ + o	+	+	+	+	+	+	+	+	+	+	0.5 c.c., dilution 1:10.
2					+	o	o	+ oo	+	+	+	+	+	+	+	+	+	+	0.5 c.c., dilution 1:10.
3					o	o	o	o	o	o	+	+	+	+	+	+	+	+	0.2 c.c., dilution 1:10.
4	Contaminated, not used																		
5	o	o	+ + o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0.3 c.c., undiluted.
6	o	o	+ + o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0.3 c.c., undiluted.
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Not used.
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Not used.

Antigens 7 and 8 were tested as high as 1.8 cubic centimeters to 0.1 of a cubic centimeter of serum against rabbit 498 which had been immunized with *M. albus,* and complement fixation was not obtained; antigens 7 and 8, therefore, were not used, since in distilled water chilling and digesting had resulted in no extraction of active antigenic fixable substance. The results were negative and it was found impossible to employ compound antigens 1, 2, and 5. Later the tests were repeated (table IX).

On June 25, seven days after the fourth and last inoculations, the rabbit sera were again tested against the compound polyvalent antigens, 1, 2, 3, and antigens 5 and 6 (table IX). The compound antigens were again found to be worthless.

TABLE IX.

Rabbit No. June 25, 1913.	*S. viridans* compound antigen.			*M. albus.*	
	1	2	3	5	6
521	+ + + + + o + + +	+	+	Antigen inhibits	+
482		o o o + o o + + +	+ + + + + o + + +	Antigen inhibits	+ + o + + o + + +
498 *M. albus*	+ o o + + + + + +	+	+	Antigen inhibits	o

On July 10, twenty-two days after the last inoculations, the rabbit sera were tested against single antigens prepared from eleven of the strains used for immunization; the results are shown in table X.

TABLE X.

Rabbit serum. July 10, 1913.	Wassermann reaction.	Gonococcus.	Antigens, *S. viridans.*										
			1	2	3	4	5	6	7	8	9	10	11
S. viridans 521	+	+	o	Antigen inhibits	o	o	Antigen inhibits	o	o	o	o	o	+ o o + + o + + o
S. viridans 482	+	+	o	Antigen inhibits	o	o	Antigen inhibits	o	o	o	o	o	o o o + o o + + o
S. viridans 244	+	+	o	Antigen inhibits	o	o	Antigen inhibits	o	o	o	o	o	o
M. albus 498	+	+	+	Antigen inhibits	+	+	Antigen inhibits	+	+	+ + + + + o + o o	+ o o + o o + o o	+ + o + + o + + +	+

SUMMARY.

1. *Streptococcus viridans* is of extremely low virulence for white mice and rabbits.

2. The serum of rabbits, after a series of intravenous injections of dead or living *Streptococcus viridans,* is poor in complement

fixative substance (*fixateur* of Besredka) for *Streptococcus viridans*.

3. Antigens prepared from several strains of *Streptococcus viridans* are relatively poor in fixable substance.

4. The technique of complement fixation tests for infection with *Streptococcus viridans* is so laborious and difficult that the method will not be available for general clinical work.

A NOTE UPON THE TECHNIQUE AND ACCURACY OF THE METHOD OF DOUGLAS AND HALDANE FOR CALCULATING THE DEAD SPACE IN BREATHING.*

By EDWARD PERKINS CARTER, M.D.

(*From The Saranac Laboratory for the Study of Tuberculosis, Saranac Lake.*)

PLATE 6.

The following notes are based upon the results obtained in a study of a group of cases of artificial pneumothorax, as employed in the treatment of pulmonary tuberculosis, of the application of the Haldane-Priestley[1] method of estimating the percentage of CO_2 in the alveolar air, and the calculation of the dead space by the method of Douglas and Haldane.[2]

No detailed report of these cases is undertaken at the present time, but in view of the recent critical analysis by Krogh and Lindhard[3, 4] of the method employed, it has seemed to the writer that certain observations, which have arisen in the course of this study, bearing upon the technique and accuracy of this method of determining the dead space, particularly in clinical cases, might be of interest.

No attempt has been made to compare the applicability of any of the other various methods of estimating the percentage of CO_2 in the alveolar air, or for the calculation of the dead space, as by Siebeck's[5] method, or by that recently described in Krogh and Lindhard's[6] basic paper.

* Received for publication, April 28, 1914.

1 Haldane, J. S., and Priestley, J. G., *Jour. Physiol.*, 1905, xxxii, 240.

2 Douglas, C. G., and Haldane, J. S., *idem*, 1912–13, xlv, 235.

3 Krogh and Lindhard, *Jour. Physiol.*, 1913–14, xlvii, 30.

4 Krogh and Lindhard, *ibid.*, p. 431.

5 Siebeck, R., *Skand. Arch. f. Physiol.*, 1911, xxv, 87.

6 Krogh and Lindhard, *loc. cit.*, p. 431.

Under the circumstances, any method involving a technique which must be carried out wholly in the laboratory was obviously impossible, as the cases studied were scattered about the village of Saranac Lake, many of them being confined to bed and only a small number being ambulatory. Fortunately the conditions under which it was necessary to carry on the observations fulfilled, in a large measure, the chief indication considered fundamental as affecting the accuracy of the Haldane-Priestley method of determining the percentage of CO_2 in the alveolar air, *i. e.*, the patients were at rest.

Krogh and Lindhard[7] have shown that the direct method of determining the composition of the alveolar air from samples taken by the Haldane-Priestley method becomes untrustworthy during muscular work, and in their later paper[8] they have emphasized this fact more fully. They also state:

> "During rest the increase in CO_2 percentage in the alveoli is not proportional to the time after the beginning of expiration, and the average of correct H.-P. inspiration and expiration samples is therefore *not* the true average tension. The difference can not be considerable, however, and we believe that in persons with a shallow respiration the H.-P. method may frequently be used with advantage as the variations in the composition of the alveolar air during the respiratory cycle must be small. There is a certain danger, however, that the respiration preceding the taking of a sample may be unconsciously but systematically altered (usually shortened) by the subject and that may vitiate the results to a great extent."

The technique of the method of procedure in this study was evolved in the endeavor to secure as great an accuracy as possible under the conditions imposed. It is self evident that the samples of alveolar air and of expired air must be secured under absolutely the same conditions, and it was soon found that the observations must be made, as far as possible, at the same hour of the day.

All the analyses have been made with Haldane's standard apparatus,[9] the samples being obtained by means of Haldane's gas sampling tubes.

[7] Krogh and Lindhard, *loc. cit.*, p. 30.
[8] Krogh and Lindhard, *loc. cit.*, p. 443.
[9] The apparatus was supplied by Baird and Tatlock, Ltd., London.

ALVEOLAR AIR.

The samples of alveolar air were obtained by the method of Haldane and Priestley, by the use of, in the earlier observations, the simple respiration tube, as originally described, with a single outlet for the alveolar air sampling tube two and a half inches from the mouth end. This tube was made from ordinary hose, and in order to overcome its tendency to curve it was reinforced by a thin brass rod wired to the outside, thus securing an absolutely straight egress and ingress for respiration.

After breathing quietly in and out through the tube, the nose being held, for a sufficient length of time to secure the normal respiratory rhythm for the individual under observation, the samples were secured at the end of a forced expiration following a normal inspiration (sample 1), and again at the end of a forced expiration following a normal expiration (sample 2). At the end of the sharp forced expiration the mouthpiece was closed by the tongue, the individual signaling by closing both eyes the instant the mouth piece was occluded, and the sample of alveolar air was taken.

It was manifestly difficult to obtain always the intelligent coöperation of the individual at the first observation, but as the observations were repeated this difficulty was overcome and the incidental error largely, if not entirely eliminated.

After samples 1 and 2 were secured, as soon as the normal respiratory balance was recovered the sample of expired air was obtained, originally by the use of Zuntz's[10] apparatus and subsequently by the use of rubber bags.

The average of the percentage of CO_2 in samples 1 and 2 was taken as the mean percentage of CO_2 in the alveolar air. It soon became evident, from a number of observations, that not infrequently the percentage of CO_2 in the alveolar air, as determined in this way (the mean of samples 1 and 2), if taken again immediately after collecting the expired air varied slightly from the percentage obtained previously. This difference was not always marked, but the percentage of CO_2 in the alveolar air taken immediately after

[10] This apparatus, as used by the writer, was made under the supervision of Dr. J. J. R. Macleod, in the Physiological Laboratory of Western Reserve University.

securing the expired air was more often found to be lower rather than higher.

Two possible explanations for this difference appeared reasonable. Inasmuch as we were dealing with individuals in whom any unusual though slight effort tended to produce a certain degree of dyspnea, it was conceivable that following the collection of the expired air the CO_2 percentage in the alveolar air was actually lowered; or it may well be that the difference between the two samples is the result of an unavoidable error in technique due to a difference in the time of the expiratory phase at which the sample was secured.

Using a four-way mouthpiece (figure 1),[11] the writer found that, in a sharp forcible expiration, the percentage of CO_2 in the alveolar air increased gradually, from that period of time as nearly as possible coincident with the end of the normal expiratory phase, up to the extreme limit of forced expiration, as follows:

	I. Percentage of CO_2.	II. Percentage of CO_2.	III. Percentage of CO_2.	IV. Percentage of CO_2.
E. P. C.	4.84	4.87	5.29	5.62
E. P. C.	4.79	5.18	5.56	5.71
E. P. C.	4.31	4.71	5.41	5.59

I = approximately just after the limit of normal expiration.
IV = approximately the limit of forced expiration.

As a result of these observations the procedure was adopted of securing, in every instance, the two samples of alveolar air both before and after collecting the expired air. By means of the four-way mouthpiece samples 1 and 2 were secured, the expired air was then collected, and without any unnecessary delay the two additional samples (3 and 4) of alveolar air were obtained, and the mean percentage of CO_2 in all four samples was taken as the average percentage of CO_2 in the alveolar air at the time of the observation (table I).

[11] This mouthpiece has four outlets placed two and a half inches from the mouth end. The sampling tubes were opened and closed by colleagues in the order 1, 2, 3, and 4, at a given signal. Other similar records are not quoted. It is, of course, apparent that the figures 1, 2, 3, and 4 do not indicate with any accuracy the same period of time in the forced expiration in the different observations; they can be only approximately correct. (Krogh and Lindhard, *loc. cit.*, p. 431. This article was not published at the time the above observations were originally made.)

TABLE I.

Case No.	Date.	Tidal air in c.c.	Alveolar air CO_2% before taking expired air.	Alveolar air CO_2% after taking expired air.	Alveolar air CO_2, mean percentage.	Alveolar air CO_2 tension.	
						In percentage of 1 atmosphere.	In mm. of mercury.
1	Jan. 15, 1914	294.3	5.73	5.51	5.62	5.61	40.1[12]
1	Jan. 19, 1914	174.6	6.02	5.98	6.00	5.65	40.4
1	Feb. 27, 1914	212.0	5.79	6.10	5.94	5.59	40.0
1	Feb. 28, 1914	281.0	6.16	5.63	5.89	5.57	39.8
1	Mar. 6, 1914	255.2	6.57	6.23	6.40	6.02	43.1
1	Mar. 7, 1914	261.0	6.55	6.26	6.40	6.02	43.1
3	Feb. 2, 1914	666.6	6.96	6.74	6.85	6.45	46.1
3	Feb. 3, 1914	728.7	7.02	6.65	6.83	6.43	46.0
3	Feb. 4, 1914	638.4	6.52	6.35	6.43	6.05	43.3
4	Feb. 18, 1914	534.2	6.16	5.82	5.99	5.64	40.3
4	Feb. 18, 1914	525.5	6.03	5.71	5.87	5.53	39.5
6	Mar. 11, 1914	569.0	5.39	5.52	5.45	5.13	36.7
6	Mar. 12, 1914	603.0	5.55	5.72	5.63	5.30	37.9

It is, of course, evident that such a four-way mouthpiece is not in any sense an essential necessity in securing the four samples. The writer adopted its use, however, as offering the greatest accuracy in standardizing his technique in the method of procedure, in avoiding any unnecessary delay after the observation was begun, and as a great convenience with bed patients. It has been used in all recorded observations since the end of January.

TABLE II.

Case No.	Date.	Tidal air.	I. Alveolar air CO_2%.	II. Alveolar air CO_2%.	III. Alveolar air CO_2%.	IV. Alveolar air CO_2%.	Alveolar air, mean CO_2%.	Alveolar air CO_2 tension.	
								In percentage of 1 atmosphere.	In mm. of mercury.
9	Mar. 31, 1914	520.7	5.40	5.51	5.09	5.34	5.33	5.02	35.9
9	Apr. 1, 1914	542.0	5.39	5.59	5.09	5.41	5.37	5.06	36.2
9	Apr. 2, 1914	602.0	5.59	5.76	5.30	5.72	5.59	5.26	37.6
Mean		554.9	5.46	5.62	5.16	5.49	5.43	5.11	36.5

I = alveolar air sample following inspiration before collecting the expired air.
II = alveolar air sample following expiration before collecting the expired air.
III = alveolar air sample following inspiration after collecting the expired air.
IV = alveolar air sample following expiration after collecting the expired air.

[12] Saranac Lake is 503 meters above sea level. In all calculations the mean barometric pressure,—based upon actual barometric observations extending over three months,—is taken at 716 mm. of mercury.

As illustrating in greater detail the point emphasized, a single example will suffice (table II), being the results of three observations on successive days before the establishment of an artificial pneumothorax.

THE EXPIRED AIR.

The collection of the sample of the expired air was at first attempted by means of the Zuntz apparatus, in the hope that it would be possible to collect data regarding the respiratory quotient, which would be more reliable than when the bag is used, but this method was given up in favor of the rubber bag, as used by Douglas and Haldane[13] and their associates, for the following reasons. In a number of individuals the sense of air-hunger induced by the Zuntz apparatus was so great that the observation had to be abandoned in a very short time. It was also found that with the Zuntz apparatus the length of time necessary to secure an accurate sample of the expired air was longer than any of the cases studied could submit to; and finally there remained the difficulty of transporting the apparatus and meter from house to house, and its inapplicability for patients in bed.

With the rubber bag it is only necessary to collect the expired air for a definite length of time, counting accurately the number of respirations during the period of observation, and then to pass the amount collected through the wet gas meter, collecting the samples into previously exhausted sampling tubes. In this way one can collect any number of samples desired. Four minutes were taken as the standard length of time for the collection of the expired air. In a number of observations this was lengthened to five minutes, and in a few instances it was found necessary to shorten the time to three minutes, but in no case did the time fall below this latter figure. In individuals with a large tidal air the duration of any such observation depends in a measure upon the capacity of the bag employed. The two bags used had a capacity of fifty-two and fifty-seven cubic liters respectively.

The bag is used with an inlet tube of wide caliber about two meters long. By constructing a valve, on the principle of the in-

[13] Douglas, C. G., Haldane, J. S., Henderson, Y., and Schneider, E. C., *Phil. Tr. Roy. Soc. London,* 1913, cciii, series B, 185–318.

take valve of the Zuntz apparatus, and using dogs' intestine as the valve proper, it was possible to provide a valve which offers the least resistance to expiration and yet effectually prevents any unconscious rebreathing or escape of the air from the bag before it is clamped off at the conclusion of the observation. The allowance for dead space when measuring the expired air is ninety cubic centimeters.

Before starting the observation the patient breathes quietly into the bag for one to two minutes, without the valve in place, and this expired air is then forced out of the bag, thus assuring that the slight amount of residual air in the bag at the commencement of the observation was the individual's own expired air.

The method of procedure was as follows: At a given signal the patient starts breathing directly into the bag through the mouthpiece of the valve described above, inspiring through the nostrils and expiring through the mouth, the nostrils being held closed during expiration; the patients were also taught to occlude the opening in the mouthpiece with the tongue during inspiration, thus automatically reinforcing the valve.

In cases 3, 4, and 9 of this series, from which a few observations reported herewith are taken, it was found impossible to occlude the nostrils during expiration, because of the sense of discomfort produced, and the failure to do this leaves the method as employed in these cases open to a certain just criticism. A number of control observations, however, with different individuals, gave a difference of less than fifteen cubic centimeters in the tidal air figure when the expired air is collected in this way, at the same respiratory rate and for the same length of time, and no difference in its analysis.

In every instance the expired air was measured within half an hour after its collection.

In using the rubber bag two precautions are necessary and of fundamental importance as affecting the accuracy of the results obtained. Before measuring the expired air the bag must be thoroughly shaken to ensure a complete mixture of its contents, otherwise the resulting analyses of the CO_2 and O_2 may be wholly inaccurate. Finally, but by no means the least important procedure in the technique of the rubber bag method, the bag should be thor-

TABLE III.

Case No.	Date.	Respirations per minute.	Tidal air in c.c.	Alveolar air CO_2%.	Alveolar air CO_2 tension.		Expired air CO_2%.	Alveolar air in c.c.	Calculated dead space in c.c.	Remarks.
					In percentage of 1 atmosphere.	In mm. of mercury.				
3	Feb. 3, 1914	17.6	729	6.83	6.43	46.0	4.24	465	264	Ambulatory, all observations sitting.
3	Feb. 4, 1914	17.5	638	6.43	6.05	43.3	4.32	428	210	
3	Mar. 20, 1914	15.0	508	6.78	6.37	45.6	4.29	327	187	
4	Feb. 4, 1914	14.0	500	6.09	5.72	40.9	4.18	343	157	Ambulatory, all observations sitting.
4	Feb. 4, 1914	15.0	551	6.19	5.83	41.6	4.27	380	171[14]	
4	Feb. 18, 1914	13.5	534	5.99	5.64	40.3	4.12	367	167	
4	Feb. 18, 1914	13.0	525	5.87	5.53	39.5	4.37	390	135[14]	
6	Feb. 11, 1914	15.4	423	5.87	5.53	39.5	4.08	327	96	Ambulatory, all observations sitting.
6	Feb. 11, 1914	18.7	413	5.21	4.90	35.0	4.12	332	81[14]	
6	Feb. 21, 1914	17.0	211	5.47	5.15	36.8	4.07	157	54	
6	Feb. 21, 1914	18.3	251	5.55	5.22	37.3	4.06	183	68[14]	
6	Mar. 3, 1914	18.3	396	5.34	5.03	36.0	4.04	301	95	
6	Mar. 3, 1914	18.3	328	5.60	5.27	37.7	4.14	242	86[14]	
9	Mar. 31, 1914	12.2	521	5.33	5.02	35.9	3.98	388	133	In bed.
9	Apr. 1, 1914	12.0	542	5.37	5.06	36.2	3.94	399	143	In bed.
9	Apr. 2, 1914	12.2	602	5.59	5.26	37.6	3.91	421	181	In bed.
9	Apr. 3, 1914	13.5	277	5.68	5.35	38.2	4.42	212	65	24 hrs. after first artificial pneumothorax.
10	Apr. 6, 1914	19.7	355	6.18	5.82	41.6	3.81	220	135	In bed.
10	Apr. 7, 1914	24.2	299	6.10	5.74	41.0	3.77	184	115	24 hrs. after first artificial pneumothorax.

[14] One hour after paracentesis for reëstablishment of artificial pneumothorax.

oughly washed out after each observation by ordinary atmospheric air, in order to prevent any accumulation of organic exhalations or the collection of pockets of CO_2 which may also give rise to inaccurate readings. This has been done by filling the bags from an ordinary laboratory bellows blower twice and then emptying completely. The writer found that unless this procedure was carried out the CO_2 percentages were not as accurate as when controlled by a control bag.

The figures in table III are taken from five of the cases studied in this series, and are chosen as being typically characteristic of the results obtained in an attempt to apply the method of Douglas and Haldane for determining the dead space in breathing in a group of individuals who fulfill to an unusual degree the requirement of bodily inactivity.

No detailed analysis of these cases is attempted at the present time; they are cited merely to add force to the points noted above in the technique of the method employed, and serve to emphasize the truth of Krogh and Lindhard's contention that:

> "When however the alveolar CO_2 percentage is used together with the corresponding percentage in the expired air for calculating the dead space of the subject, even small deviations from the true composition of the air expired from the alveoli may have a very considerable effect upon the reliability of the result."

It must be borne in mind that all the individuals in this series are the subjects of a more or less advanced pulmonary tuberculosis, E. P. C. alone being entirely free from the disease. This fact explains the high percentage of CO_2 in the expired air.[15]

TABLE IV.

Four Control Observations Made on E. P. C. at Weekly Intervals.[16]

Respirations per minute.	Tidal air in c.c.	Alveolar CO_2%.	Alveolar air CO_2 tension.		Expired air CO_2%.	Alveolar air in c.c.	Calculated dead space in c.c.
			In percentage of 1 atmosphere.	In mm. of mercury.			
17	783	5.62	5.29	38.0	3.55	496	287
16	934	5.59	5.26	37.6	3.22	538	396
17	753	5.68	5.35	38.3	3.29	429	324
17	751	5.54	5.21	37.3	3.27	443	308

[15] The actual analyses were all carefully checked by duplicates.

[16] The writer has a large tidal air capacity, and a very constant respiratory rate. It is perhaps unnecessary to add that all the observations have been made on adults.

Turning to the figures given in tables III and IV, it is evident that in those instances in which the tidal air exceeds 423 cubic centimeters the calculated dead space is out of all true proportion to the alveolar air. In only one instance, observation 4, case 4, in which the tidal air is above this figure, does the calculated dead space for the individual approach what is probably the normal or true dead space for the individual at rest, and here the CO_2 percentage in the expired air rose abruptly following the paracentesis for the reëstablishment of the pneumothorax.

I wish, in concluding, to express my great indebtedness to Dr. Edward R. Baldwin, without whose interest and aid these observations would have been impossible, as they have been made almost entirely upon his private cases. To Dr. J. J. R. Macleod, of Western Reserve University, to whom I owe my introduction into the method of the physiological technique, my appreciative thanks are due. The study from which these brief notes are taken has been carried out entirely under Dr. Macleod's guidance. I am under deep obligations to him for many valuable suggestions and his friendly interest in the problems encountered.

CONCLUSIONS.

The determination of the percentage of CO_2 in the alveolar air, by the method of Haldane and Priestley, is sufficiently accurate for clinical purposes when the individual is at rest.

It is evident, however, that an error may arise in the determination of the percentage of CO_2 in the alveolar air, due to the time during the forced expiration, short of the extreme limit, at which the sample may be unconsciously taken. This error can only be overcome by taking several samples.

In individuals at rest having a tidal air below 425 cubic centimeters, the method of Douglas and Haldane for the calculation of the dead space gives approximately accurate results; with a tidal air above 425 cubic centimeters the results obtained may be wholly inaccurate.

In patients with marked pulmonary tuberculosis so slight an effort as that involved in collecting the expired air tends to lower the percentage of CO_2 in the alveolar air.

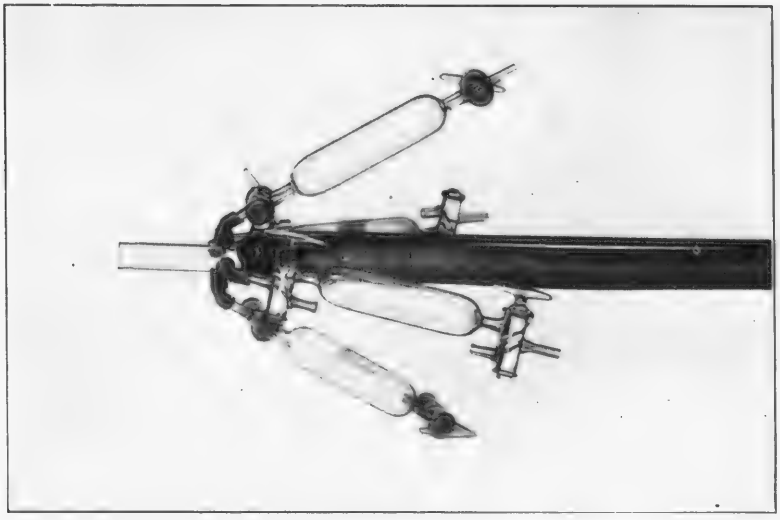

FIG. 1.

(Carter: Calculation of Dead Space in Breathing.)

In collecting the expired air by the rubber bag method, it is absolutely essential that the bag be washed out with ordinary atmospheric air after every observation.

EXPLANATION OF PLATE 6.

FIG. 1. The four-way mouthpiece used in the experiments. For further details, see footnote 11, page 84.